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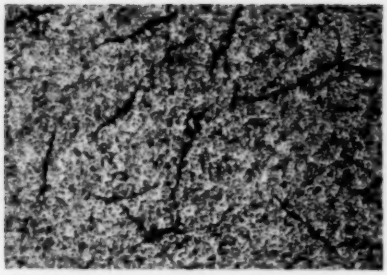

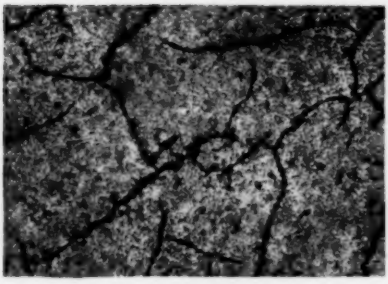

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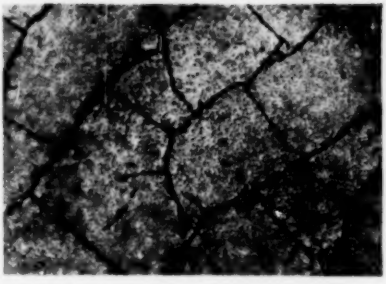

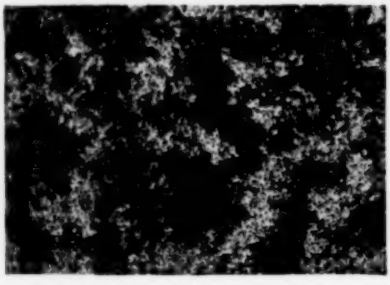

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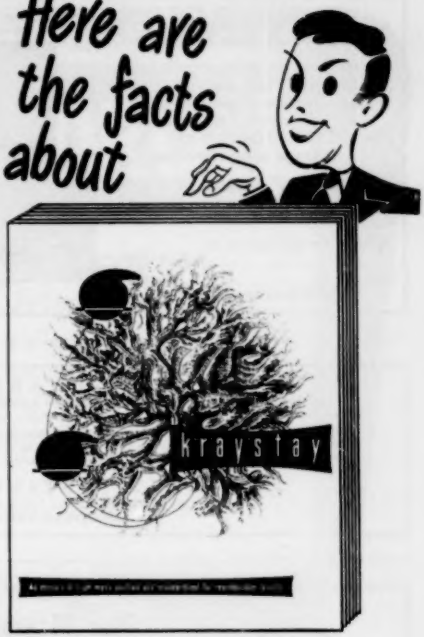

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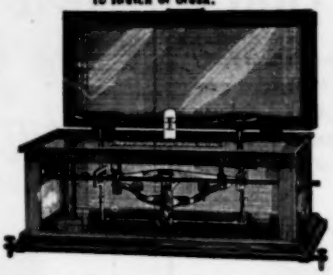

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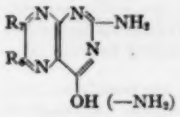
Paper Chromatography of Some Synthetic Pteridines*

By ALICE G. RENFREW and PAULINE C. PIATT

Partition chromatography on filter paper was utilized as a means for qualitative identification of some synthetic pteridines, mainly substituted isoxanthopterins. In general, separations of 4-hydroxy and 4-amino analogs were possible. R_F values for xanthopterin, dihydroxanthopterin, and leucopterin were determined with the use of butanol-morpholine-water or of 3 per cent aqueous ammonium chloride as developing solvents.

BECAUSE of the great contrast in physiological action between pteroylglutamic acid and aminopterin (termed a folic antagonist), we have prepared two series of substituted pteridines: 4-hydroxypteridines and the 4-amino analogs of this series. Among other methods of characterizing the two series, partition chromatography on filter paper was tested. The rate of movement of these pteridine derivatives is markedly influenced by the substituents; for some pairs the hydroxy and amino analogs are readily separated by paper chromatography. Xanthopterin (I), dihydroxanthopterin, and leucopterin were included in the studies. R_F values for two of the latter substances have already been reported by Good and Johnson (1) with the use of chromatograms developed with butanol-acetic acid (2). Hirata, *et al.* (2), cited the results of paper chromatography as part of the evidence for the isolation of xanthopterin from skins of the yellow mutant of *Bombyx mori*.



I. Xanthopterin: R_6 = OH; R_7 = H
II. Isoxanthopterin: R_6 = H; R_7 = OH

Interest in the synthetic isoxanthopterin (II) structures is increased by the fact that isoxanthopterin appears to be the end product when folic acid is exposed *in vitro* to the successive action of ultraviolet irradiation and of "xanthine oxidase" (3). In the preparation of the synthetic pteridines reported in the present paper, 4,5-aminopyrimidines were condensed with various α-keto esters; e. g., ethyl benzoylformate, ethyl oxalacetate, triethyl oxalsuccinate, and isoamyl pyruvate. In the resulting pteridines, the 2 position was occupied by $-NH_2$, and the 4 position by —OH (as in folic acid) or by $-NH_2$ (as in aminopterin). Condensations were carried out by refluxing in normal acetic acid under conditions (4, 5) favoring the formation of isoxanthopterins (II). Evidence for the presence of the acetic acid group in the 6 position was provided by decarboxylation in boiling 2 *N* hydrochloric acid to

* Received July 19, 1950, from the Department of Research in Pure Chemistry, Mellon Institute, Pittsburgh, Pa.

6-methylisoxanthopterin. The resultant 6-methyl derivative in solution in 0.1 *N* hydrochloric acid showed maxima at 290 and 335 mμ; in close accord with the values of 290 and 336 mμ as given by Elion and Hitchings (6). Russell, *et al.* (7), also mention this method for obtaining 6-methyl-isoxanthopterin. With some of these condensation products the full proof of structure is still in progress. When condensations were carried out in 0.25 *N* hydrochloric acid (5) to favor the formation of 7-substituted xanthopterins (I), the product was characterized both by the R_F value given in Table I, and by one or more components of higher R_F. These products are under investigation.

EXPERIMENTAL

The present study of two series of pteridines gives evidence that R_F values[1] are influenced by the group in the 4 position, and that these values differ appreciably in relation to the substituents in the 6 and 7 position. *n*-Butanol-morpholine-water (3:1:3 by volume) has been a convenient solvent mixture in the development of one-way chromatograms. In this solvent combination, the presence of a 4-amino substituent or of an aromatic group in the pyrazine ring seems to favor rapid movement; a carboxylic substituent in the pyrazine ring appears to retard the movement of the test spot. Similar development rates were obtained with the solvent mixture recommended by Vischer and Chargaff (9) for the study of purines and pyrimidines. Low R_F values were found in using butanol-water in the presence of ammonia as described in the study of purines and pyrimidines by Hotchkiss (10). Markham and Smith (11) comment concerning purines, "in the presence of ammonia the amino derivatives move faster than the corresponding hydroxy compounds."

R_F values, cited in Table I, are usually an average of five or more determinations. Although in hot weather these values were higher numerically, the relative rates of movement of different compounds were not altered (2). The phenyl derivative was frequently carried as a reference standard (2) on chromatograms because this compound gave spots with an intense blue fluorescence. In artificial admixtures of the 4-amino analog and the corresponding 4-hydroxypteridine, clear-cut separations were evidenced by the pair of 6-methyl analogs and the pair of phenyl analogs inasmuch as the chromatogram with butanol-morpholine-water showed two spots with R_F values which checked the concurrent development of the individual components. For derivatives containing carboxyl substituents, the R_F values were too similar to permit separation of the analogs by the butanol-morpholine-water; separation did occur on chromatograms developed with aqueous 3% ammonium chloride.

In *n*-butanol-acetic acid-water (8:1:1), chromatograms of the pteridines required long development to sharpen the spots, and this solvent gave less satisfactory separation of artificial mixtures of 4-hydroxy and 4-aminopteridines, as might be anticipated from values shown in Table I. With the use of the two phases of the solvent mixture described by Partridge (2), the R_F values were numerically higher but a separation of analogs was not effected. With 3% aqueous ammonium chloride, the solvent front traveled very rapidly (35 cm. in four hours); for the synthetic pteridines many R_F values were exceedingly low and the spots diffuse, although methyl derivatives developed rather well. Phenol-water (3:1) showed some promise in the development of pteridines with a carboxyl-substituent, as tested with the acetic acid side chain and the folic acid oxidation product (12). However, methyl and phenyl substituted pteridines moved almost as fast as the solvent front in phenol water, and fluorescence was faint. Butanol phenol-water mixtures gave R_F values <0.2 and sometimes fluorescence was completely absent.

Xanthopterin.—On chromatograms of very dilute solutions (10 mg./ml. of 3 *N* NH_4OH) we were unable to differentiate between xanthopterin and the chemical precursor, dihydroxanthopterin, as indicated in Table I; leucopterin traveled more slowly. Solutions of xanthopterin, after forty-eight hours or more of storage at room temperature, showed a faint spot in a position corresponding to leucopterin.

Strong yellow fluorescence was seen on chromatograms spotted with one drop of the more concentrated solution which contained 0.1 Gm. of xanthopterin in 100 ml. of 0.5 *N* ammonium hydroxide (1). With ascending chromatography

[1] R_F = mm. traveled by the fluorescent band per mm. traveled by the solvent past the starting line (8, 10).

TABLE I.—R_F VALUES OF SOME SYNTHETIC PTERIDINES

Substituents in Position 6[a]	R_F Butanol-Morpholine-Water 4-OH	R_F Butanol-Morpholine-Water 4-NH_2	R_F Butanol-Acetic-Water 4-OH	R_F Butanol-Acetic-Water 4-NH_2
—CH_2COOH	0.32	0.31 (and 0.47)	0.13	0.10
—$CH(COOH)CH_2COOH$[b]	0.33[b]	0.28[b]	0.10	0.13
—CH_3	0.38	0.44	0.18	0.26
—C_6H_5	0.50	0.63	0.41	0.42
Xanthopterin[c]	0.5 –0.6			
Dihydroxanthopterin[c]	0.5 –0.6			
Leucopterin[c]	0.25–0.3			

[a] The 7 position carries an hydroxyl group, except in the case of the xanthopterins.
[b] The ethyl ester was not stable under these conditions, but gave approximate values of 0.70 and 0.80 for the 4-OH and 4-NH_2 structures, respectively.
[c] On chromatograms developed with 3% aqueous ammonium chloride, xanthopterin, and dihydroxanthopterin had average R_F values of 0.68, and leucopterin an R_F value of 0.30. A sample of riboflavin, dissolved in the presence of acetic and boric acids, was developed for comparison with xanthopterin and gave an R_F value of 0.4.

there was difficulty in resolving the fluorescent stream to the area of discrete spots. When morpholine was present in the developing solvent, the yellow fluorescence of the moist chromatogram was seen as a blue fluorescence on the dry sheet. A 3% aqueous solution of ammonium chloride permitted five to six hour development of xanthopterin and dihydroxanthopterin, but the fluorescence was a pale yellow-green which made the spots difficult to outline sharply. Leucopterin gave a very faint spot.

PROCEDURE

The R_F values in Table I were determined by ascending solvent movement (13) with the use of cylinders of Whatman No. 1 filter paper. For the test spots, solutions of 1 mg. of pteridine in 100 ml. of 3 *N* ammonium hydroxide were prepared and three small drops were placed on the sheet (one drop equals approximately 0.02 ml.). The cylinders were strengthened by a cuff at the bottom and top (14) and the vertical edges were tied together with glass thread to avoid corrosion of metal clips. The butanol-morpholine-water solvent front traveled about 21 cm. in fifteen hours, and the butanol-acetic acid-water, 32 cm. in twenty-three hours in summer weather. Papers were air-dried and the fluorescent spots were observed and marked under a Hanovia ultraviolet lamp.

SUMMARY

1. Some synthetic 4-hydroxy and 4-aminopteridines have been studied by partition chromatography on filter paper. The fluorescent spots were observed under ultraviolet light.

2. R_F values, determined with the use of *n*-butanol-morpholine-water (3:1:3 by volume) for the developing solvent, show the 4-amino derivatives as moving more rapidly than the 4-hydroxy analogs in general. For derivatives containing a carboxyl substituent, aqueous 3 per cent ammonium chloride brought out a similar difference.

3. Brief observations on the development with other solvent mixtures are noted.

4. Xanthopterin, dihydroxanthopterin, and leucopterin gave approximate R_F values of 0.5, 0.5, and 0.23, respectively, on development with butanol-morpholine-water. With 3 per cent ammonium chloride, the respective R_F values averaged 0.7, 0.7 and 0.3.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Norephedrine
6,7-Dichloro-9-ribityl-isoalloxazine
D-Epigalactose
Vulbocapnine
Galactoflavin
Tetracosane
alpha-Bromoisocaproyl bromide
Hemimellitic anhydride
2,3-Dichlorodiphenylamine
2-Methyl-5-*n*-butylpyridine
1,4-Dimethylimidazole
N-Methyl thiopropionamide
Butylthiamin
Phenylhydroxamic acid
D-Primario acid
Triuret
meta-Aminohippuric acid
Primula acid
Phytolaccinum
Renin

A Quantitative Colorimetric Method for Certain Ketosteroids Using a Modification of the Zimmerman Reaction*

By ROBERT P. TANSEY† and JOHN M. CROSS‡,§

A colorimetric procedure for the determination of certain ketosteroids by the use of 3,5-dinitrobenzoic acid and beazyl trimethyl ammonium hydroxide is described. Experimental evidence of its adaptability to the determination of estrone is presented. The maximum absorption of the color complex with estrone occurs at 530 to 535 mμ. The extinction coefficient ($E^{1\ cm.}_{1\%}$) is 170. Beer's law is followed through a range of 20 to 200 μg. of estrone. The stability of the color and the elimination of the need for anhydrous conditions are advantages of the method.

A NUMBER of color reactions pertaining to ketosteroids, particularly estrogenic substances are described in the literature (1–11). It is apparent that the existing procedures require very exacting techniques before reliable end points and reproducibility of results can be attained. One of the most widely used chemical methods is the Zimmerman reaction (12,13) as modified by Callow, Callow, and Emmens (14). Escamilla (15) used this procedure for clinical evaluation of urinary hormonal assays. Nathanson and Wilson (16) preferred the Zimmerman technique to other colorimetric methods and stated there was less technical difficulty involved and a smaller increment of error present.

The original Zimmerman reaction involves the treatment of a ketosteroid with *m*-dinitrobenzene in the presence of an alcoholic solution of potassium hydroxide. A color is formed which reaches its maximum intensity in sixty to eighty minutes. The reaction can be used for 17-ketosteroids, such as androsterone and estrone. The absorption maximum of the color complex varies with the position and nature of the carbonyl group. Consequently, this test is of diagnostic value. The color complex with 17-ketosteroid shows a maximum at about 520 mμ and the unsaturated 3-ketosteroid color complex has a band of intensity at 380 mμ (12). A 20-ketosteroid gives a color intensity about one-fifth that of the 17-ketosteroid (12). This reaction is also positive with certain saturated sterols and bile acids containing 3-keto groups. The test is reported to be negative when the keto group is in the 6, 7, or 12 position (12).

The reaction between a ketone and a nitrocompound of benzene in the presence of an alcoholic solution of potassium hydroxide was first reported by Janovsky in 1886 and 1891 (17). He referred to various color reactions with acetone and several nitrobenzene compounds. Von Bitto in 1892 (17) cited the same type of color complex, using ketonic substances other than acetone.

Wu and Chow (18) described a modification of the Zimmerman method and used it in determining the endocrine activity of urine extracts. Oesting (19) and Oesting and Webster (20) used the Zimmerman technique on various estrogenic substances and subsequently attempted to correlate their data with biological assays on the same material. Callow, Callow, and Emmens (14) used a modification of the Zimmerman reaction in their assay procedure. They also made biological assays in conjunction with their chemical determinations in obtaining information which would be useful in the development of a suitable estrogenic assay method. Pesez and Herbain (21) reported on differences in reactivities of the carbonyl groups with certain steroid compounds using a modified Zimmerman reaction.

The Zimmerman reaction is very sensitive to alkali. The preparation of the reagents also presents some difficulties. The *m*-dinitrobenzene is difficult to purify and the absolute alcohol that is used must not have more than a trace of aldehydes present. Metallic inorganic alkalies re-

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act with the nitrobenzene compound to give a brown color. In order to find a more desirable procedure and one in which the color is more stable and more easily developed, an experiment was initiated using 3,5-dinitrobenzoic acid in place of the *m*-dinitrobenzene (22). This compound was tried because of its solubility in aqueous alkaline solution. Thereby, the need for organic solvents and anhydrous conditions can be circumvented. It was found, however, that this compound is also very sensitive to inorganic alkalies, turning brown in the presence of sodium or potassium hydroxides. In order to overcome this tendency, an organic alkali, benzyl trimethyl ammonium hydroxide, was tried (22). The color which occurs when the organic alkali is mixed with the 3,5-dinitrobenzoic acid is completely discharged upon dilution with water and a colorless solution was obtained. The test solution must be freshly prepared since it tends to darken on standing.

When a 17-ketosteroid and 3,5-dinitrobenzoic acid are dissolved in a 40 per cent solution of benzyl trimethyl ammonium hydroxide, a purple color is immediately produced which follows Beer's law under prescribed conditions.

The specificity of the reaction was investigated and it was found that a color complex is formed with various ketosteroid substances such as estrone, progesterone, androsterone, testosterone, and dehydrocholic acid. Nonketonic substances such as estradiol gave negative results. The color complexes with these ketosteroids show absorption maxima at different wave lengths in the visible range of the spectrum.

Pure estrone (U. S. P. Reference Standard) was used in attempting to develop a suitable assay procedure using this modification of the Zimmerman reaction. The technique involved is simple and the color developed is stable for at least one hour. The results are reproducible and the method can be used over a wide range of estrone concentrations.

EXPERIMENTAL

Materials Required

Alcohol.—Methyl alcohol, ethyl alcohol, or 2B specially denatured alcohol can be used as solvents for estrone.

3,5-Dinitrobenzoic Acid.—Eastman Kodak Company, white label grade, is further purified by recrystallization from ethyl ether. It has a straw yellow color and melts at 204°.

Benzyl Trimethyl Ammonium Hydroxide.—A 40% aqueous solution of this material was obtained from Rohm and Haas and is known as Triton-B, 40% aqueous solution.

Reagent.—A 0.1% solution of 3,5-dinitrobenzoic acid in 40% aqueous benzyl trimethyl ammonium hydroxide. This reagent should be freshly prepared.

Procedure

A standard solution of estrone in methyl alcohol is prepared (10 mg./50 ml.). Aliquots of this solution are subdivided into clean test tubes. The test tubes are then placed in a suitable apparatus (Fig. 1) and the contents brought down to dryness on a boiling water bath. Nitrogen is passed through the test tubes during the drying period. When the residue is completely dried, after about ten minutes, the test tubes are transferred to a cold water bath (15 to 20°). They are then detached from the drying apparatus. At this point 0.5 ml. of the freshly prepared reagent is added to each tube.

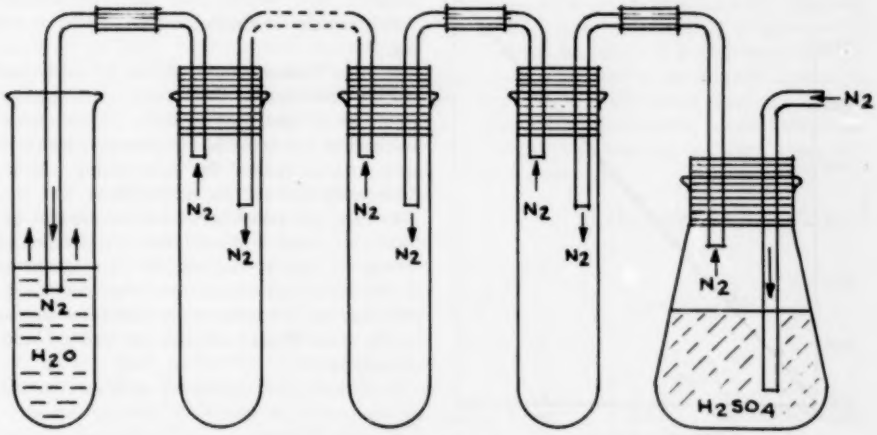

Fig. 1.—Drying apparatus.

A blank is prepared using the reagent without the sample material. After thorough mixing they are allowed to remain at room temperature for ten minutes. Ten milliliters of distilled water is then added to each tube and the contents again thoroughly mixed. Readings are taken on the photoelectric colorimeter or spectrophotometer after the samples have stood for an additional ten minutes.

The maximum absorption band as determined on the Beckman Model DU and Coleman Model 11A spectrophotometers and on the Evelyn photoelectric colorimeter is shown in Fig. 2. The maximum intensity occurs at 530 to 535 mμ on the Beckman and 530 mμ on the Coleman. A 515 filter gave a maximum reading with the Evelyn. A Beer's law curve was prepared using the Beckman unit (Fig. 3). The stability of the color complex (Fig. 4) was evaluated on the Evelyn colorimeter using a 515 filter.

The extinction coefficient (E) is calculated to be approximately 170 for estrone.

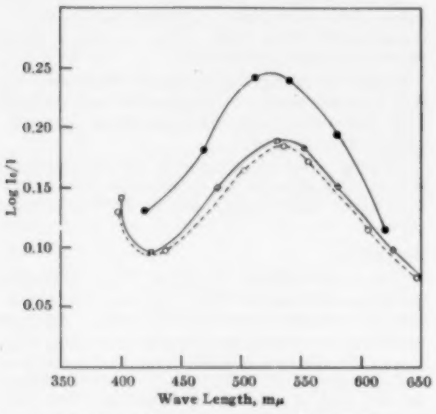

Fig. 2.—Absorption spectra of estrone.
Evelyn: 80 micrograms/final solution (●).
Beckman Model DU: 110 micrograms/final solution (□).
Coleman Model 11A: 110 micrograms/final solution (○).

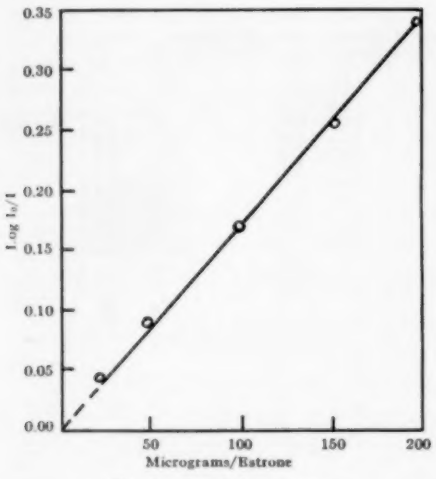

Fig. 3.—Beer's law curve.

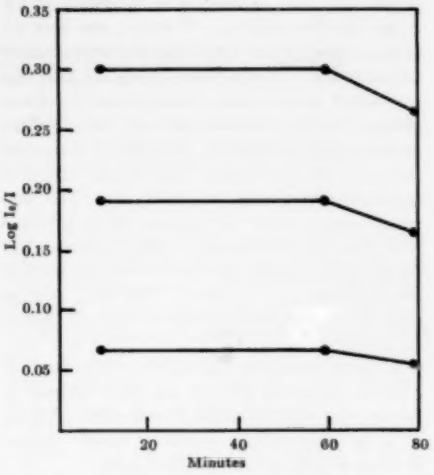

Fig. 4.—Stability of color complex with estrone.

SUMMARY

1. A quantitative colorimetric method of analysis for an estrogenic ketosteroid is given in detail. The reaction appears to be common for ketosteroids with the carbonyl group at the 3-, 17-, or 20-positions and the assay technique may be applicable to most of these compounds.

2. The absorption spectrum of the colored complex with estrone has been determined. The maximum absorption occurs at 530 to 535 mμ.

3. The method described can be easily and quickly performed. The color developed is stable for at least sixty minutes. Readings can be checked and rechecked within that period of time without fear of the color fading. Anhydrous conditions need not be prescribed.

4. The procedure is specific for certain ketonic substances and each can be identified in certain regions of the visible color spectrum. A cursory examination shows that the 3,5-dinitrobenzoic acid forms color complexes similar to the *m*-dinitrobenzene with the various ketosteroids tested.

5. Graphs are presented showing the absorption curve and Beer's law curve for estrone.

6. The procedure was applicable over a range of 20 to 200 μg. with estrone.

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A Comparison of a New Astringent Agent with Such Agents Now Commonly Used*

By JOHN E. CHRISTIAN and GLENN L. JENKINS

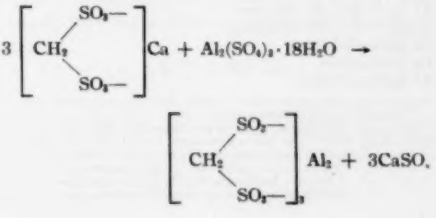
The synthesis of a new astringent, aluminum methionate, is reported. Extensive comparative protein precipitation tests for astringency indicate that this salt is more effective as an astringent than other common astringent agents tested, and thus may be applied to improve perspiration retarding or inhibiting preparations. Cloth tests indicate that aluminum methionate is harmless to fabric.

ASTRINGENTS are used therapeutically for a variety of purposes including the treatment of diarrhea, the reduction of inflammation of mucous membranes, the promotion of healing, and as antihemorrhagic agents. The effects produced are probably due to protein precipitation and hardening of the cell surface producing contraction and decreasing the permeability of the capillaries (1). Astringent agents, because of their ability to reduce secretion and exudation, are also used as antiperspirants.

A number of antiperspirants have been proposed to control or retard the flow of perspiration from certain skin areas by application of suitable preparations of astringent material. The more common types of preparation for this purpose contain an acid salt of a metal, usually aluminum chloride, aluminum sulfate, zinc chloride, aluminum nitrate, alum, and similar salts. Such substances are effective in stopping perspiration but are unsatisfactory and hazardous in that the use thereof frequently produces acute skin irritation or dermatitis and rots clothing fabrics which come in contact with the treated areas. This action has been shown to be due primarily to the acid radical.

In this laboratory a large number of known astringent agents were tested for astringent power and for their effect on cloth and the results compared with the values obtained using an astringent agent previously unreported. This work was done in an effort to provide a new and improved astringent agent which combines full inhibitory perspirant action equivalent to, or better than, other aluminum salts, but avoids their corrosive action and is harmless to the skin and fabrics. It was found that the utilization of one of a series of aluminum alkyl disulfonates, namely aluminum methionate, was superior to other commonly used aluminum salts.

EXPERIMENTAL



* Received April 24, 1950, from the research laboratories of the Purdue University School of Pharmacy, Lafayette, Ind.

Calcium methionate (100 Gm.) was dissolved in 250 cc. of warm water and 102 Gm. (98% of theory) of crystalline aluminum sulfate, dissolved in 250 cc. of water, was added slowly with stirring. The mixture was digested for ten minutes, cooled to room temperature, filtered, and the calcium sulfate washed with water. This water was added to the original filtrate. The filtrates may be concentrated, an analysis made, and the aluminum methionate solution used as such. The pure salt was isolated as follows: the aluminum methionate solution was concentrated until a supersaturated solution was obtained. Alcohol was added, causing the aluminum salt to crystallize out. Further purification was made by recrystallization from water and alcohol. Yield: 87 Gm. (96% of theory).

Anal.—Calcd. for $C_9H_6S_9O_{18}Al_2$: Al, 9.38%; S, 33.33%. Found: Al, 9.25%; S, 33.39%.

Properties.—Aluminum methionate is somewhat hygroscopic and is quite soluble in water, forming a 69% solution w/v at 27°. The *p*H of a 5% aqueous solution is about 3.5.

Astringent Tests.—Few works are reported in the literature concerning the measurement of relative astringency. Auch (2) mentioned the use of animal skin for measurement of astringency. Sollmann (3, 4) reports the utilization of the precipitation of proteins as an indication of astringency. Theis (5) has described a method involving a "Bottle Dilatometer."

In this laboratory, preliminary testing methods indicated the protein precipitation tests to be more reliable and reproducible. A number of proteins, including egg albumin, casein, washed dried blood fibrin (Armour), fibrin from blood (Sargent), albumin from blood (bovine) (Armour), and powdered blood albumin (Armour) were used in preliminary protein precipitation tests with some of the common astringent agents. Egg albumin was found to give uncertain and nondifferential results since its composition changes from sample to sample. Blood albumin and fibrin from various sources were found to be unsatisfactory since consistent results were not obtained and since precipitation occurred irrespective of relative concentrations of astringents. In addition, some astringent agents such as formaldehyde do not affect these proteins. Hammerstein's casein was found to have a uniform composition and to give good results. The testing procedure was carried out as follows: a 5% solution of Hammerstein's casein (100 cc.) was prepared by shaking the casein to a uniform suspension with 50 cc. of 0.1 *N* HCl. Additional HCl was added and the mixture heated on a steam bath until solution resulted. After cooling, the solution was brought up to volume with additional 0.1 *N* HCl. To 5-cc. portions of the casein solution, 1 cc. of each astringent was added slowly with shaking. The mixture was incubated for fifteen minutes at 37°, cooled, and centrifuged. A portion of the supernatant liquid was removed and tested for complete protein precipitation by the addition of three drops of 25% $AlCl_3$ solution. Table I and Table II give the results of these tests using various astringents. According to these tests aluminum methionate has the greatest ability to precipitate protein. Aluminum methionate was also shown to be superior to aluminum chloride and aluminum sulfate by blood agglutination techniques.

On a molar basis, a 1 molar solution of $AlCl_3$ contains the same amount of aluminum as a 0.5 molar solution of $Al_2(SO_4)_3$ and aluminum methionate. As in the case of percentage solutions, aluminum methionate is shown to be a superior protein-precipitating agent.

The Effect of Aluminum Methionate on Fabric

Relative noncorrosive tests were made on cloth with different astringent preparations. The procedure used was "The Good Housekeeping procedure for testing deodorants as to safety on fabrics."

TABLE I.—CASEIN PRECIPITATION ABILITY OF SOME ASTRINGENT AGENTS

Substance	Concentration, % 3	5	8	10	15	20	25
$AlCl_3$	+−−−	++−−	+++−	++++	....	++++	++++
$Al_2(SO_4)_3$	+−−−	++−−	+++−	+++−	....	++++	++++
Al Methionate	++−−	+++−	++++	++++	++++	++++	++++
Al Chloral Hydrate Complex	−−−−	−−−−	−−−−	....	....	+−−−	+−−−
Al Sulfoacetate	−−−−	−−−−	−−−−	−−−−	....	+++−	....
Al Ethane Disulfonate	+−−−	++−−	++++	++++	....	++++	....
Al *o*-Sulfobenzoate	−−−−	+−−−	+−−−	++−−	....	+++−	....
Al Nitrate	....	+−−−	+++−	++++	++++	++++	++++
Al Ethyl Sulfate	....	−−−−	....	−−−−	++−−	+++−	....
Lead Nitrate	....	....	+−−−	....	....	....	....
Stannous Chloride[a]	....	....	+−−−	....	....	....	....
Zinc Nitrate	....	....	+++−	....	....	....	....
Lead Acetate[a]	....	....	+−−−	....	....	....	....
Zinc Acetate[a]	....	....	+++−	....	....	....	....
$AlCl_3$ + 8% Zn Acetate	....	....	+−−−	....	....	....	....
$AlCl_3$ + 8% Pb Acetate	....	....	−−−−	....	....	....	....
Zn Sulfocarbolate	....	....	+−−−	++−−	....	++++	....
Zn Sulfanilate	....	....	+−−−	+−−−	....	+++−	....
Al Sulfocarbolate	....	....	+−−−	++−−	++++	++++	....

[a] Supersaturated solutions.

++++ Indicates complete precipitation of protein, as judged by the absence of any change on addition of three drops of a 25% $AlCl_3$ solution to the supernatant liquid obtained after centrifugation.

+++− Indicates almost complete precipitation as shown by cloudiness on addition of the $AlCl_3$ solution to the supernatant.

++−− Indicates precipitation in the supernatant on addition of the $AlCl_3$ solution.

+−−− Indicates further precipitation in the supernatant.

−−−− Indicates very heavy precipitation in the supernatant and no precipitation by the astringent tested.

TABLE II.—CASEIN PRECIPITATION ABILITY OF ALUMINUM CHLORIDE, SULFATE, AND METHIONATE COMPARED ON A MOLAR BASIS

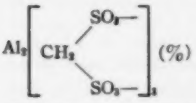
Substance	Concentration (Molar) 1	0.5	0.25	0.125	0.0675
$AlCl_3 \cdot 6H_2O$ (%)	24.14	12.07	6.03	3.01	....
	++++	++++	++−−	−−−−	....
$Al_2(SO_4)_3 \cdot 18H_2O$ (%)	....	33.32	16.66	8.33	4.16
	....	++++	+++−	+−−−	−−−−
 (%)	....	28.8	14.4	7.2	3.4
	....	++++	++++	++++	+++−

TABLE III.—BREAKING LOADS OF TREATED CLOTH (POUNDS)

Cloth Number 1	2	3	4	5	6	7	8	9	10	11	12	13
72	69	65	65	72	59	10	12	56	39	50	50	51
68	70	62	67	61	65	5	20	51	35	55	52	46
69	63	61	66	65	64	6	13	55	36	66	41	49
71	71	66	70	58	65	7	14	55	50	56	52	48
63	66	65	57	67	67	6	14	54	53	56	51	42
62	62	59	60	66	62	11	12	62	36	58	49	41
61	66	62	67	64	61	7	20	50	21	54	51	40
61	76	64	68	68	65	10	11	49	27	65	52	39
67	69	60	70	61	59	10	14	45	39	60	54	50
64	71	60	74	62	62	10	20	52	30	58	50	40
66	60	66	65	57	69	9	18	55	17	58	55	36
66	67	59	62	65	64	5	20	45	21	61	50	36
63	73	62	68	63	70	7	15	51	27	61	48	40
75	64	69	70	64	71	11	20	49	38	58	54	46
67	68	72	72	63	67	9	15	55	30	57	52	39
Av. 66.3	67.6	62.7	66.1	63.7	64.7	8.2	15.8	52.6	33.2	58.2	52.3	42.8

Ordinary bleached muslin was pressed and cut into strips 6 x 21 in. Down the center of each strip, over an area 1 in. wide, was applied 1 Gm. of the cream in question. The strips were then held in an incubator at 38° for forty-eight hours, and at the end of this time pressed uniformly, both as to time and temperature, with an electric ironer. The strips were then cut into five pieces 6 x 4 in. These pieces were allowed to stand for twelve hours, and then broken on a standard Tensile Strength Machine by the Grab Test (Scott Yarn Tester). The front jaws were 1 in. wide and the back jaws 2 in. wide. The distance between the upper and lower jaws was 3 in. The speed of pulling the jaws was 12½ in. per minute. Three of the long strips were treated with each product giving 15 breaking strength determinations. All tests were run simultaneously.

The average breaking loads in Table III indicate that cloth is weakened very little, if any, by creams containing high concentrations of aluminum methionate, whereas commercial astringent preparations show in each case considerable weakening of cloth.

Cloth No.	Specimens Tested for Breaking Load Treatment
1	Untreated control
2	Cream base control
3	25% aluminum methionate in cream base
4	25% aluminum methionate and 8% urea in cream base
5	15% aluminum methionate in cream base
6	15% aluminum methionate and 8% urea in cream base
7–13	Commercial astringent creams and liquids A, B, C, D, E, F, G

SUMMARY

The synthesis of a new astringent, aluminum methane disulfonate (aluminum methionate), is reported. Extensive comparative protein precipitation tests for astringency indicate that this salt is more effective as an astringent agent than other common astringent agents tested, and thus may be applied to improve perspiration retarding or inhibiting preparations. Comparative cloth tests indicated that aluminum methionate is harmless to fabric, whereas other common astringent agents tested were found to weaken cloth considerably. The salt has the additional advantage in that it is very soluble, hygroscopic, and nontoxic. The salt being hygroscopic aids in preventing the drying-out and hardening of creams containing it. Extensive tests (6) have shown that the methionate radical is nontoxic.

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A Chromatographic Study of the Anthraquinone Derivatives of Curacao Aloe*,†

By THEODORE M. BRODY, RALPH F. VOIGT, and FRANK T. MAHER

Existing chromatographic methods have been successfully adapted to the isolation and the purification of the anthraquinone derivatives of Curaçao aloe. Two trihydroxy-methylanthraquinones, aloe-emodin and iso-emodin, have been separated from extracts of Curaçao aloe. The use of chromatography as a quantitative method for the anthraquinone drugs has been verified and a quantitation of the total anthraquinone content of Curaçao aloe is proposed.

NUMEROUS INVESTIGATORS have sought to establish a method of analysis for the several species of aloe. These have included procedures for the chemical assay of the constituents of the drug as well as biological studies seeking to quantitate the cathartic activity. Tilden (1), Shafer (2), Eder and Schneiter (3), and Briggs (4) have based their analyses on the determination of the water-soluble glycosides. Colorimetric methods of analysis of the anthraquinone drugs have been described by Valaer and Mallory (5), Katti and Beal (6), Tschirch and Heipe (7), Hebeisen (8), Jorgensen (9), and Carol (10). Chemical studies have been hampered by differences of opinion concerning the constituents and their distribution in various aloe species. The constituents of the drug responsible for the cathartic action have not been completely identified. Several workers have shown that the purgative activity may be due to the anthraquinone derivatives (11, 12). Kiefer (13), Wirth and co-workers (14), and Viehoever (15) have attempted to correlate chemical constitution with drug activity. In general, biological testing methods have been unreliable. Due to these difficulties, the official quality of aloe is established on the basis of the water-soluble extractives.

Zechmeister and Cholnoky (16) have described the work of Ernst and Weiner who employed the chromatographic method in the identification of anthraquinone drugs. This enabled them to make a comparative study of these drugs. However, they did not isolate nor analyze the individual chemical constituents. In 1947, Gibson and Schwarting (17) successfully isolated and analyzed the trihydroxy-methylanthraquinones of cascara sagrada, separating three isomers on a magnesia-celite column.

The present investigation has made use of chromatographic separation and spectrophotometric analysis in studying the anthraquinone derivatives of Curaçao aloe.

EXPERIMENTAL

A number of adsorbing agents have been used for the anthraquinone drugs, including aluminum oxide, bentonite, kaolin, magnesia, and a mixture of magnesia and celite (17). In this work a mixture of one part of magnesia[1] and three parts of Celite[2] gave the best chromatographic differentiation on prepared adsorption columns. Pyrex tubes 25 cm. in length and 17 mm. in inside diameter were used as chromatographic tubes. Adsorption columns were prepared in these tubes by placing a piedget of glass wool in the bottom of the column and filling the tube uniformly with the adsorbent. A layer of glass wool was placed on the top of the adsorbing agent. The column was then connected to a 500-cc. separatory funnel with an airtight cork connection. Uniform adsorption and a clear differentiation of layers were facilitated by careful packing of the magnesia-celite mixture in the columns.

Extraction.—The trihydroxy-methylanthraquinones from powdered Curaçao aloe were extracted by a modification of Daels' method (18). Five grams of powdered drug was placed in a 500-cc. round-bottomed flask, 250 cc. of chloroform was added, and the mixture was refluxed for one hour. The resulting yellow extract was then filtered. The filter paper containing the residue was returned to the original container and refluxed for two and one-half hours with another 250 cc. of chloroform and 50 cc. of 25% sulfuric acid. The resulting chloroform solution was separated from the aqueous layer. This method yielded two chloroformic extracts. The first, a pale yellow solution, contained the free anthraquinones present in the drug. The second extract, a dark orange-colored solution, contained the anthraquinones freed by acid hydrolysis from the "combined" form present as glycosides in the original drug.

Chromatography.—Chloroformic extracts of the free anthraquinones and of the hydrolyzed anthraquinones were allowed to flow through chromato-

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[1] Westvaco Adsorptive Magnesia No. 2641, Westvaco Chlorine Products Company.
[2] Celite No. 501, Johns-Manville Company.

graphic columns prepared as previously described. Five hundred cubic centimeters of extract of either type produced a chromatogram characterized by a nondifferentiated dark red layer.

The presence of anthraquinone derivatives in this dark red layer was confirmed by treating the extruded red layer with 10% hydrochloric acid, extracting the residue with chloroform, and examining the chloroformic extracts in the Beckman Quartz Spectrophotometer. The transmittancy curves prepared by the usual procedures (Fig. 1) revealed peaks of maximum absorption at 440 mμ and at, or below, 320 mμ. Stone and Furman (19) have shown that dilute solutions of trihydroxy-methyl-anthraquinone derivatives exhibit maximum absorption at approximately 450 mμ.

On the hypothesis that the lack of differentiation in the dark red layer was a function of concentration, a series of chromatograms were prepared using chloroformic extracts further diluted with chloroform. Such dilution with seven volumes of chloroform produced chromatograms characterized by four discrete layers, as shown in Table I. This dilution of the original chloroform extracts was, therefore, adopted for all analyses.

TABLE I.—CHROMATOGRAMS OBTAINED BY VARYING CONCENTRATIONS OF CHLOROFORM EXTRACTS OF CURAÇAO ALOE FREED FROM THE COMBINED STATE BY ACID HYDROLYSIS

Solution 1 Undiluted	Solution 2 1:1	Solution 3 1:3	Solution 4 1:7
Brown-black layer, 4 mm.	Red-brown layer, 10 mm.	Red-brown layer, 9 mm.	Red-brown layer, 4 mm.
Dark red layer, 54 mm.	Bright red layer, 26 mm.	Bright red layer, 17 mm.	Bright red layer, 8 mm.
Yellow layer, 20 mm.	Pink-tan layer, 10 mm.	Pink-orange layer, 7 mm.	Pink layer, 2 mm.
	Yellow layer, 10 mm.	Yellow layer, 6 mm.	Yellow layer, 2 mm.

Chromatograms so prepared consistently exhibited the red-brown, the bright red, the pink, and the yellow layers. These columns were extruded and the separated layers were eluted with 10% hydrochloric acid and extracted with chloroform. Transmittancy curves were prepared from the chloroformic extracts (Fig. 2).

Extracts of each of the red and pink layers showed maximum absorption at approximately 440 mμ. Extracts of the yellow layers again exhibited maximum absorption at or below 320 mμ. The curve obtained from the eluate of the pink layer was poorly defined and indicated admixture with material from the yellow layer. The red-brown layer was also eluted and subsequent analysis showed it to be a mixture of red and pink layers.

The presence of anthraquinone derivatives was confirmed by applying the Borntrager reaction to chloroform extracts of the eluted layers as described by Fairbairn (20). Extracts of eluates from the red layer gave a positive Borntrager reaction with both sodium hydroxide and ammonium hydroxide test solution. Extracts of eluates from the pink layer gave a positive reaction with sodium hydroxide solution and a negative reaction with ammonia.

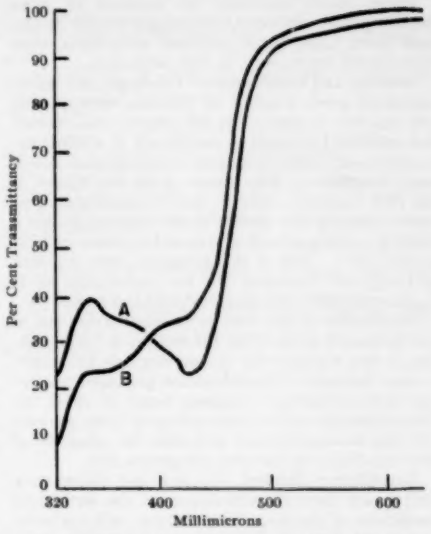

Fig. 1.—Transmittancy curves of (*A*) chloroformic solution of eluates, and (*B*) chloroformic extracts of Curaçao aloe.

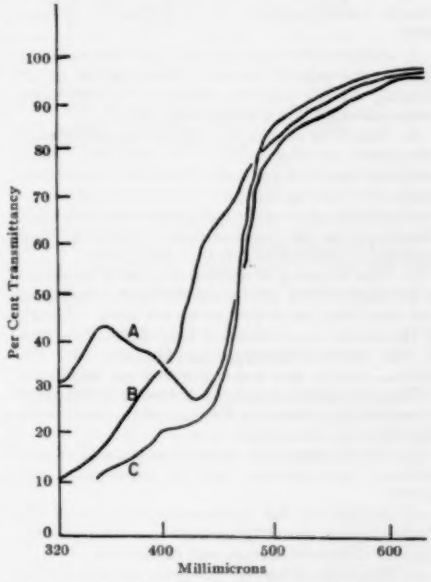

Fig. 2.—Transmittancy curves of chloroformic solutions of eluates from (*A*) red layer; (*B*) yellow; and (*C*) pink layer, of Curaçao aloe chromatograms.

These results postulate the presence of aloe-emodin in the red layer, and of iso-emodin in the pink layer. Additional isolation procedures were then carried out to support this conclusion.

Isolation and Purification.—The bright red layers separated from a series of columns were pooled and allowed to dry. The red powder so obtained was purified by repeated procedures of elution extraction and chromatographic adsorption as previously described. The extract from the eluate of the fifth successive column was evaporated to dryness. Heating the residue under reduced pressure yielded a sublimate of orange-red crystals melting at 217–218°. This is in agreement with the data of Cahn and Simonsen (21) for aloe-emodin or 3-(hydroxymethyl)-1-8-dihydroxyanthraquinone.

Purification of the pink layer was carried out in the manner described for the red layer. The sublimate was washed with carbon disulfide to remove a waxy impurity. Resublimation produced orange-red crystals having a melting point of 181–183°. This conforms to the observations of other workers (17) for iso-emodin and indicated the presence of this constituent in the aloe samples studied.

Quantitative Studies.—Gibson and Schwarting (22) based their determination on the extinction coefficient of emodin, the principal anthraquinone compound isolated from cascara sagrada. Since Curaçao aloe had yielded aloe-emodin in greatest quantity, the feasibility of quantitating the total anthraquinone content on the basis of the aloe-emodin extinction coefficient was examined. Use of this procedure is supported by the finding that both aloe-emodin and iso-emodin exhibit maximum absorption at 440 mμ. The following experiments were performed to confirm the quantitation of the yellow color of anthraquinones in chloroform solution as a reliable means of evaluation for Curaçao aloe.

1. Solutions of aloe-emodin and iso-emodin as well as the yellow material (characterized as anthranol) on the column conformed to Beer's law when examined spectrophotometrically.

2. Solutions of mixtures of known quantities of iso-emodin and aloe-emodin were prepared. These contained identical amounts of total anthraquinones made up of varying amounts of the two constituents. Spectrophotometric examination revealed only slight differences in the transmittancy values of these solutions at maximum spectral absorption.

3. The amounts of anthranol present in eluates to be assayed were minimized by closely separating and discarding the yellow anthranol band. Eluates of the yellow band exhibited only slight absorption in the spectrophotometer at 440 mμ, and this minimal interference was considered not significant.

These considerations have led us to recommend a method of evaluating Curaçao aloe, based upon the following principles:

(*a*) Chromatographic separation of total anthraquinones (aloe-emodin and iso-emodin) as described.

(*b*) Elution of the anthraquinone band of the chromatogram by 10% hydrochloric acid and extraction of anthraquinone with chloroform.

(*c*) Spectrophotometric analysis at 440 mμ of the chloroformic extracts for free and combined anthraquinone, against a standard curve of aloe-emodin isolated as described.

(*d*) Anthranol interference is minimized by discarding the anthranol layer of the chromatogram, and by the low spectral absorption of anthranol at 440 mμ.

DISCUSSION

Gibson and Schwarting (17) have reported that aloe-emodin and emodin in cascara sagrada extracts give rise, respectively, to orange and red bands on their adsorbing columns. Our study of this apparent difference in the color of aloe-emodin on similar columns has led to the conclusion that higher dilutions of aloe-emodin produce orange bands when adsorbed on columns containing lower proportions of magnesia. This would indicate that aloe contains more aloe-emodin than does cascara and that the orange band reported by Gibson and Schwarting is due to this lower content of aloe-emodin in their cascara samples.

Stone and Furman (19) proposed a polarographic method for the estimation of aloe-emodin and the aloins in Curaçao aloe. Their analysis of aloe-emodin was based upon the premise that a chloroform extract of aloe removes only aloe-emodin. The results of the polarographic method are compared with a colorimetric method based on the absorptance of a chloroform extract of Curaçao aloe. Our studies have shown the presence of two components (iso-emodin and the anthranols) in addition to aloe-emodin in chloroform extracts of aloe. It was also possible to isolate these same compounds on a chromatographic column from chloroform extracts of aloin. This result is not surprising since aloin is usually prepared from Curaçao aloe.

Of the constituents obtained by chromatographic isolation, iso-emodin, aloe-emodin and the anthranols, it seems logical to separate the first two compounds from the anthranols. Since no accurate quantitative method has been devised to separate these isomers, this investigation favors the analysis of total anthraquinone present in the drug, based upon the common point of maximum spectral absorption of aloe-emodin and of iso-emodin.

CONCLUSIONS

The present study of the anthraquinone derivatives has sought to clarify the existing differences of opinion regarding the nature of the principal chemical constituents. Experimental data have led to the following conclusions:

1. Iso-emodin, aloe-emodin, and anthranols have been isolated from Curaçao aloe. These have been shown to exist in the free state and in combination from which they may be readily freed by acid hydrolysis.

2. The differentiation of an extract of Curaçao aloe on a chromatographic column of magnesia-celite is a function of the concentration of that extract and the composition of the adsorbent mixture.

3. Under identical conditions, extracts of aloin exhibit chromatograms similar to those of Curaçao aloe. Socotrine and Cape aloe extracts

could not be satisfactorily differentiated on the chromatographic column used.

4. A previous spectrophotometric method for the quantitation of extracts of anthraquinone drugs has been successfully adapted for the analysis of total anthraquinone in aloe based upon the extinction coefficient of aloe-emodin.

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The Influence of Varying Nitrogen Levels on Hydroponic Growth and Alkaloid Production in *Hyoscyamus muticus* L.*,†

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This investigation describes the successful hydroponic growth of *Hyoscyamus muticus* L., using varying concentrations of nitrogen in Skok media. Maximum development and growth were observed between 252 and 336 p.p.m. Analysis of the flowering plants revealed constant moisture content of the total plant and its component parts at nitrogen supplies above 42 p.p.m. Fresh and dried plant weights reached a maximum at 252 p.p.m. and remained constant to 672 p.p.m. of nitrogen. Increasing the nitrate-nitrogen content of the supplied media from 0 to 252 p.p.m. resulted in an absolute increase in total alkaloid production. The flower tops contained the greatest concentration of alkaloid.

FIELD STUDIES on the cultivation of *Hyoscyamus muticus* L. were first reported in 1949 by Ahmed and Fahmy (1), who first recognized the potential value of the high alkaloid content of this plant.

Prior to the publication of their results by these authors, preliminary investigation in this laboratory indicated the feasibility of cultural studies of *Hyoscyamus muticus* L. by the use of hydroponic methods. Under these conditions the plants are readily propagated and retain their naturally occurring high alkaloid content.

The present investigation has been directed to a study of the influence of varying nitrogen supply on the growth and production of alkaloid by *Hyoscyamus muticus* L., using hydroponic procedures.

EXPERIMENTAL

Selected plants were grown in hydroponic jars filled with clean quartz. Nutrient solutions were prepared to provide a range of known nitrogen supply. These solutions contained the following amounts of nitrate-nitrogen:

No. 1.....	0 p.p.m. of nitrogen
No. 2.....	42 p.p.m. of nitrogen
No. 3.....	84 p.p.m. of nitrogen
No. 4.....	168 p.p.m. of nitrogen
No. 5.....	252 p.p.m. of nitrogen
No. 6.....	336 p.p.m. of nitrogen
No. 7.....	420 p.p.m. of nitrogen

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No. 8	504 p.p.m. of nitrogen
No. 9	588 p.p.m. of nitrogen
No. 10	672 p.p.m. of nitrogen

The essential elements in these nutrient solutions were made up of various salts according to the formula used by Skok (2) in 1941. By choosing various combinations of salts, it was possible to alter the nitrate-nitrogen control and still maintain a constant ratio of the other elements. An incompatibility with the iron salts was overcome by the use of iron ammonium citrate as suggested by Reed and Haas (3) in 1924. The ammonium-nitrogen from this salt did not significantly alter the total nitrogen in each solution. The hydrogen-ion concentration ranged from 5 to 5.3 for each of the prepared solutions.

In order to facilitate germination, seeds of *Hyoscyamus muticus* L. were treated with concentrated sulfuric acid for one and one-half minutes. This step was immediately followed by a quick and thorough washing with water until free of acid, after which the seeds were air-dried.

Prior to sowing the seed, the no. 2 quartz sand in each of the one-gallon glazed earthenware jars was cleaned with hydrochloric acid and then washed free of the acid with distilled water. The jars were arranged for the ten different treatments with nitrogen, with each series replicated four times.

Several seeds were sown at a depth of one-fourth inch in each jar. During the germination period the quartz medium was kept moistened with distilled water and the daily average temperature was 63° F.

Eleven days after germination one typical seedling was selected in each jar and the remaining ones were discarded. These seedlings were all of the same vigor and height At this time the use of distilled water was discontinued and the prepared nutrient solutions were applied. These plants were watered periodically, either every twenty-four or forty-eight hours, depending upon the environmental temperatures and the development of the plant.

A modified flooding method was employed to feed the plants. Each of the nutrient solutions from an elevated container was siphoned into the basal opening of the hydroponic jar. When the solution appeared above sand level, the siphon was disconnected, allowing the excess liquid in the sand to drain gradually. Each watering required approximately 250 cc. per jar.

From the time the seedlings appeared until the time of harvest, the plants were grown under the same environmental conditions. During this period of sixteen weeks, the average daily temperature was kept between 63 and 70° F. The total sunlight amounted to 602 hours or an average of 5.4 per day. The relative humidity remained fairly constant at 60%.

From the ninth week of this investigation until the time of harvesting, observations were made of each plant weekly: height and breadth of plant, average leaf size, leaf coloration, extent of branching, time of blooming, and number of flowers.

As soon as the plants began to bloom, they were examined systematically and verified as *Hyoscyamus muticus* L. The plants were harvested sixteen weeks after the sowing of the seeds, and final observations were made at this time.

Each plant was harvested individually. The above-ground part was divided into leaf, stem, and flowering top. The root systems were freed of adhering sand by immersion and agitation in water. The excessive moisture was then removed with paper toweling. The respective plant parts were weighed immediately and kept separated during the remainder of the experiment.

The fresh material was then transferred to the laboratories and air-dried at room temperature, approximately 25° C., on open-screened racks. Within six weeks the plant parts were considered to be adequately air-dried. They were then weighed, ground to a no. 20 powder, and stored in glass containers.

Analysis.—Duplicate samples of each air-dried powder were dried to constant weight by the U. S. P. XIII (4) method for crude drugs with constituents nonvolatile at 100° C.

The moisture-free material was employed in the determination of alkaloids according to the method of Brown, Kirch, and Webster (5) with the following modifications. In the first step, a 2-Gm. sample instead of 10 Gm. was extracted with 2 cc. of stronger ammonia test solution, U. S. P., and 4 cc. of ether, U. S. P., instead of 10 cc. and 20 cc., respectively. Secondly, a 50-cc. aliquot instead of 10 cc. of the extractive was employed in the chromatographic separation of the alkaloid. In the final titration of alkaloid, the 0.02 *N* sulfuric acid containing the alkaloids was back-titrated with 0.02 *N* sodium hydroxide to an end point determined electrometrically.

In a preliminary study of electrometric titration, titration curves were prepared for various known solutions of hyoscyamine. End point *p*H values from these curves indicated that increasing concentrations of hyoscyamine are associated with decreasing end point *p*H values, at concentrations below 15 mg. %.

In order to establish the end point of choice for the titration of the alkaloidal extracts encountered in this investigation, alcoholic stock solutions were prepared to contain 1 mg. and 4 mg. of hyoscyamine[1] per cc. at 20° C.

From these stock solutions, aliquots representing 5, 10, and 40 mg., respectively, of alkaloid were evaporated to dryness in 50-cc. beakers. Residues were taken up with 15 cc. of 0.02 *N* sulfuric acid and back-titrated with 0.02 *N* sodium hydroxide. The alkali was added in portions of 0.5 cc. each and the *p*H of the resulting solution was determined after each addition with the aid of a glass electrode *p*H meter. An alkaloid-free sample served as a control.

With these results, titration curves were drawn and the end points for hyoscyamine were determined as follows: 0 mg. of alkaloid, *p*H end point of 6.95; 5 mg. of alkaloid, *p*H end point of 6.60; 10 mg. of alkaloid, *p*H end point of 6.40; 40 mg. of alkaloid, *p*H end point of 6.10.

Since the amount of alkaloid present in the samples analyzed in this study varied from 5 to 10 mg., all samples were titrated to an end point *p*H of 6.5.

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[1] Merck & Company, Rahway, N. J.

Results.—During the early stages of growth, the plants developed very slowly. Beginning with the ninth week of this experiment, weekly observations revealed measurable differences in plant height. Concurrently, a number of descriptive details were recorded pertaining to the size and color of the leaves, extent of branching stems, date of blooming, and number of flowers.

Each of the first three levels of nitrogen, including 84 p.p.m., produced significant increases in plant height. The maximum height attained at this level was maintained at 168 p.p.m. Plants grown at higher nitrogen levels were consistently smaller but showed a distinct reduction in plant height at 672 p.p.m. of nitrogen.

Maximum leaf size was attained at 336 p.p.m. of nitrogen and optimum green color of the foliage was reached at the level of 252 p.p.m. Higher levels of nitrate-nitrogen did not alter these growth features. Nitrogen levels of 252 and 672 p.p.m. produced a fairly constant maximum amount of branching. Further growth effects are noted in that the plants grown in 504, 588, and 672 p.p.m. of nitrogen began to bloom one week earlier than those grown at the lower nitrogen levels. The average number of flowers per plant rapidly increased with the first five levels of nitrogen and then remained constant beginning at 336 p.p.m.

Average fresh and dry weights of plant parts grown in varying levels of nutrient nitrogen are summarized in Table I and in Fig. 1. The average alkaloid content of these plant parts is shown in Table II. These data indicate that, by all of the criteria considered, a plateau of production has been reached at nitrogen levels of 168 to 252 p.p.m. Progressively increasing the supplied nitrogen to this point has resulted in an increased production of plant tissue and alkaloid. A statistical comparison of data beyond this point, using a "p" value of 0.02 as the lower limit of probable significance, has led to the conclusion that variations beyond this maximum point are not significant. Average moisture content for all samples above 42 p.p.m. varied between 90% and 95% of fresh weight.

TABLE I.—AVERAGE WEIGHT IN GRAMS OF PLANT PARTS AT VARYING LEVELS OF NUTRIENT NITROGEN (FOUR REPLICATIONS)

Treatment	Nitrogen, P.p.m.	Leaf	Stem	Fl. Top	Root	Total
			A. Fresh Weights			
1[a]	0	...	...	...	...	...
2	42	73 ± 20.9[b]	27 ± 6.1	8 ± 5.1	21 ± 14.0	129 ± 33.6
3	84	178 ± 28.5	86 ± 10.4	21 ± 11.0	51 ± 8.8	336 ± 31.1
4	168	166 ± 41.6	112 ± 39.4	34 ± 9.0	86 ± 4.9	398 ± 83.6
5	252	287 ± 44.8	121 ± 20.2	37 ± 20.8	108 ± 31.3	553 ± 93.9
6	336	284 ± 34.5	126 ± 14.2	27 ± 6.3	90 ± 15.2	527 ± 53.9
7	420	275 ± 12.8	122 ± 4.7	39 ± 6.3	82 ± 14.4	518 ± 7.4
8	504	244 ± 26.2	109 ± 11.2	46 ± 23.2	76 ± 17.7	475 ± 62.4
9	588	249 ± 22.4	86 ± 6.1	55 ± 22.9	95 ± 8.8	485 ± 38.9
10	672	268 ± 28.7	80 ± 3.9	51 ± 23.2	88 ± 16.9	487 ± 39.5
			B. Dry Weights			
1[a]	0	...	...	...	...	...
2	42	3.6 ± 0.9[b]	1.7 ± 0.5	0.7 ± 0.3	1.6 ± 0.6	7.6 ± 1.2
3	84	9.1 ± 1.8	5.1 ± 1.2	1.4 ± 0.7	4.8 ± 1.7	20.5 ± 1.9
4	168	9.7 ± 2.0	6.4 ± 1.9	2.6 ± 0.4	7.6 ± 0.6	26.3 ± 3.5
5	252	14.1 ± 1.8	7.7 ± 1.6	2.8 ± 1.4	5.2 ± 0.7	29.8 ± 4.9
6	336	14.1 ± 1.3	7.5 ± 0.9	2.0 ± 0.3	4.2 ± 0.4	27.9 ± 1.8
7	420	13.7 ± 0.8	8.8 ± 0.7	3.0 ± 0.3	4.8 ± 0.7	30.3 ± 1.4
8	504	12.5 ± 0.5	8.4 ± 0.8	3.5 ± 1.4	4.6 ± 0.9	28.6 ± 3.5
9	588	12.4 ± 1.1	6.8 ± 0.3	4.1 ± 1.8	4.9 ± 0.4	28.2 ± 1.5
10	672	14.6 ± 1.3	6.4 ± 0.5	3.9 ± 1.5	5.2 ± 0.5	30.3 ± 0.9

[a] Samples insufficient for weighing—minimal growth.
[b] Standard deviation.

TABLE II.—AVERAGE WEIGHT OF ALKALOID IN MILLIGRAMS PER 100 GM. OF PLANT PARTS AT VARYING LEVELS OF NUTRIENT NITROGEN (FOUR REPLICATIONS)

Treatment	Nitrogen, P.p.m.	Leaf	Stem	Fl. Top	Root	Total
1[a]	0	...	...	...	...	...
2	42	150 ± 2.8[b]	180 ± 3.7	280 ± 5.9	390 ± 14.7	247 ± 29.2
3	84	260 ± 14.5	210 ± 3.7	520 ± 7.0	230 ± 8.3	305 ± 34.6
4	168	180 ± 2.5	210 ± 4.2	470 ± 10.2	170 ± 2.9	260 ± 26.5
5	252	410 ± 10.3	370 ± 6.1	910 ± 13.8	420 ± 14.2	525 ± 96.5
6	336	330 ± 10.6	310 ± 6.3	570 ± 7.1	430 ± 3.3	418 ± 58.2
7	420	330 ± 8.0	320 ± 9.2	860 ± 25.1	400 ± 10.0	496 ± 129.9
8	504	370 ± 6.3	340 ± 3.3	810 ± 14.8	480 ± 8.7	502 ± 86.6
9	588	440 ± 12.7	400 ± 5.0	970 ± 19.2	570 ± 18.7	592 ± 45.5
10	672	390 ± 2.0	350 ± 8.0	830 ± 2.8	540 ± 3.9	526 ± 26.9

[a] Minimal growth—samples insufficient for analysis.
[b] Standard deviation.

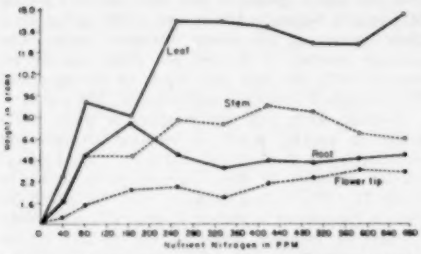

Fig. 1.—The average dry (100° C.) plant weights of plant parts at varying levels of nutrient nitrogen.

It should be noted that this normal moisture content has been achieved at levels of nutrient nitrogen inadequate for maximum growth.

DISCUSSION

Preliminary studies in this laboratory indicated that *Hyoscyamus muticus* L. grows luxuriantly under hydroponic conditions, when supplied with essential elements in the following ratios: calcium, 1.7; nitrogen, 1.2; potassium, 1.2; phosphorus, 1.0; magnesium, 1.4; and sulfur, 1.0. The accord of this experimental balance with the plant's normal fieid requirements is indicated by the plant's ability to produce blooms, and by the normal development of plants and plant parts, when grown in sand supplied with typical nutrient media. The criteria of biological activity selected to evaluate plant growth included leaf size, total plant weight, plant height, blooming time, root development, number of flowers, extent of branching, and alkaloid production.

The results of this investigation indicate the need of adequate nitrogen levels for the optimum growth of *Hyoscyamus muticus* L. From the employed criteria it is further noted the plant growth increases to an optimum, beyond which further nitrogen levels establish a plateau. It should be noted that these plants have attained optimum growth at nutrient nitrogen levels of approximately 252 p.p.m. when supplied with constant ratios of other elements, as indicated in this investigation. Further studies will be required to postulate variations in nitrogen requirements with changing ratios of other factors essential for growth.

Ahmed and Fahmy (1,6) in 1949 indicated that alkaloid content increases with increasing nitrate fertilizer and also varies in the different plant parts. Our determinations have shown that increasing nutrient nitrogen is effective in increasing alkaloid production and plant growth only up to a certain point. Above this point a plateau is established. We do not know the absolute amount of nitrogen taken up by the plants under these varying nitrogen conditions.

The determination of alkaloid content in plants studied in this investigation indicates that there is an intimate relationship between increasing nitrogen supply and increased metabolic activity and alkaloid production. The increase in alkaloid content is in part an absolute increase which is expressed in milligrams per cent of alkaloid determined. These findings may indicate a fundamental role for alkaloids in the biology of *Hyoscyamus muticus* L., since under conditions of increased nitrogen supply the formation of alkaloid has exceeded a constant ratio to plant weight. Highest alkaloid concentrations have consistently been found in the flowering top.

Further studies of alkaloid-bearing plants may provide an insight into the role of alkaloid in plant metabolism. Ahmed and Fahmy (1,6) have shown that in *Hyoscyamus muticus* L., alkaloid content increases with age to a maximum at the time of blooming, and subsequently decreases. Hughes (7) reported a sharp drop in the alkaloid content of this plant after the ripening of seed. These findings indicate that alkaloid metabolism may be involved in these vital processes.

Data here presented show an increased development of flowering parts, of high alkaloid content, and a shortened flowering period, at higher levels of nutrient nitrogen. These findings support the importance of controlled nitrogen supply in continued investigations of alkaloid in relation to plant physiology.

CONCLUSIONS

Results in this investigation have indicated the feasibility of studying the growth of *Hyoscyamus muticus* L. under hydroponic conditions and have led to the following conclusions:

1. Ascending nutrient nitrogen levels in Skok media result in an increase of the fresh and dry plant weights, reaching a maximum of 252 p.p.m. of nitrogen and thereafter remaining relatively constant to 672 p.p.m.

2. Nitrogen levels between 252 and 336 p.p.m. are associated with maximum plant height, leaf length, leaf color, extent of branching, number of flowers per plant, and root development.

3. Increasing the nutrient nitrogen content of the supplied media has resulted in an increased production of total alkaloid, to a maximum at approximately 252 p.p.m. This is in part an absolute increase, as indicated by an increase in the per cent of alkaloid in the tissue analyzed. The greatest concentration of the alkaloid is found in the flowering tops.

4. The importance of controlled nitrogen supply to further studies of alkaloid metabolism in plant physiology is discussed.

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Fungicidal Testing of Some Organo-Copper Compounds*,†

By JOHN A. SCIGLIANO, THOMAS C. GRUBB,‡ and DONALD E. SHAY§

A preliminary antimycotic test was performed using the Schamberg-Kolmer method as modified by McCrea. The results from this test suggested possible errors which might be corrected. The sources of these errors were analyzed and appropriate corrections were made. Eight organo-copper complexes with salicylic acid as the control were tested. With the exception of salicylic acid, their activity was found to be quite feeble. Variations in the *p*H of the dilutions of the compounds had no visible effect on their antimycotic activity. The actual testing technique, evolved from testing of the organo-copper complexes with salicylic acid as control, is described and it is suggested that the method would be satisfactory for testing other compounds for antimycotic activity.

MANY satisfactory methods for testing therapeutic agents have been developed for bacteria, algae, and other microorganisms, but a reliable procedure for the testing of antimycotic compounds has not yet been devised.

In recent years the increasing recognition by the medical profession of the importance of mycotic infections has led numerous investigators to center their attention upon the comparative value of antimycotic agents available. None of the techniques suggested have adequately met all of the requirements. This may be due to the controversy as to the test organism to be used, the mode of introducing the inoculum, the form of the test organism (spores, mycelia, or both) and the media to be employed. Varieties of techniques have been utilized in the preparation of the suspension and washing of the organisms involved.

In view of this lack of uniformity, it was decided to study some of the suggested tests with particular attention to the method of Schamberg and Kolmer (3) as modified by McCrea (1). Certain improvements have been offered in the work reported here, which it is felt will make the test more useful in evaluating antimycotic agents. This modified test, with which organo-copper complexes were tested against *Epidermophyton interdigitale*, *Trichophyton gypseum*, and *Megatrichophyton roseum*,[1] appears to be suitable for testing other fungicides.

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Presented to the Scientific Section, A. PH. A., Atlantic City meeting, May, 1950.

† This paper is based on a thesis presented to the Graduate School, University of Maryland by John A. Scigliano in partial fulfillment of the requirements for the degree of Master of Science in Bacteriology.

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[1] This organism was found to be the *Fusarium*, *T. mentagrophytes*, not a pathogen.

Among the first investigators to consider the therapy of fungus infections were Bokorny (2) and Schamberg and Kolmer (3). They used similar procedures. The latter workers determined the fungicidal activity of a number of compounds against *Trichophyton rosaceum*, *Microsporon audouini*, and *Achorion schoenleini*. The organisms were suspended uniformly in sterile normal saline. Equal volumes of inoculum and compound dilutions were mixed. The results indicated that benzoic and salicylic acids were poor inhibitors. These workers also tested for fungistatic properties of the same compounds. The organisms were inoculated on Sabouraud's medium to which had been added the compound dilutions.

Myers and Thienes in 1925 (5) introduced a modification into the method necessary for testing volatile oils and stearoptens. Both liquid and solid media were employed with agitation of the compound and organism mixtures between subculture intervals.

Kingery and Adkisson (6) used the same basic method, controlled the organism count in the inoculum, and determined that thymol was active.

Workers continued modification of the Schamberg-Kolmer method until 1929. At this time Reddish (7) introduced a system which, he suggested, more nearly simulated the clinical conditions met in practice. Several workers: von Gutfeld (8), Woodward, Kingery, and Williams (9, 10), Klarmann, Shternov, and Gates (11), Maplestone and Dey (12), and Stovall, Pessin and Almon (13), used the same technique on additional compounds.

Emmons (15) Gomez-Vega (16), Hasseltine and Hopkins (17), and Dunn (18) suggested the phenol coefficient method. This last does not lend itself readily to testing antimycotic substances.

EXPERIMENTAL

The compounds used in this investigation were organo-copper complexes synthesized by Whaley and Starkey (19). It was believed that, since copper and some of its compounds have shown algicidal (20), bactericidal (21), and fungicidal (20) properties, these compounds would exhibit pronounced fungicidal activity. The chemical names and formulas for these compounds appear in Table I.

A preliminary experiment was performed using the method of McCrea (14). A stock solution, 1:250 concentration, was prepared of each compound. The solvent was distilled water. The solutions were sterilized by Seitz filtration (22). From this stock solution subsequent dilutions of 1:500, 1:1,000, 1:2,000 and 1:5,000 were prepared. The *p*H values of the dilutions were recorded in view of the findings of Hoffman *et al.* (23), (see Table II). Hoffman and his co-workers showed the activity of benzoic and salicylic acids to depend on the *p*H.

The medium selected was Sabouraud's formula for glucose agar. Kolle flasks containing this medium were inoculated with the fungi by streaking the surface with an inoculating needle. After fourteen to eighteen days' incubation in the dark at 20°, the growth was removed by washing the surface with sterile distilled water. Adhering particles of growth were gently removed with a sterile loop. Matted filaments and clumps were removed by straining through 2 layers of sterile cheese cloth (No. 40 mesh), and the inoculum was standardized by comparison with the No. 10 MacFarland nephelometer tube (2).

Sterile 1-cc. tuberculin syringes were filled with the spore and mycelium suspension, and at intervals of one minute, ten minutes, one hour, and twenty-four hours, 0.5 cc. of the suspension was inoculated into tubes containing 2 cc. of the compound dilution. At the end of the designated time intervals, a loopful of this mixture was transferred to a Sabouraud's agar slant and a dextrose broth tube. Results were read after two weeks' incubation at 20°.

There was no growth after the two weeks' incubation. After a second two weeks' incubation no growth appeared. Although the results appeared to indicate high antimycotic activity, we chose to determine whether these findings were due to (*a*) an inadequate number of spores, (*b*) the presence of biological waste products, (*c*) the medium, or (*d*) the age of the organisms. In an attempt to remedy the first point cited above, we inoculated the fungi on Sabouraud's conservative medium (24), Czapek-Dox medium (25), and a modified Czapek-Dox medium (26). Daily microscopic and macroscopic studies were made of the growing culture to determine the age at which the greatest number of spores appeared. The modified Czapek-Dox medium gave the greatest concentration of spores in the shortest growing time.

TABLE II.—POTENTIOMETRIC RESULTS AT 20°

Compound	*p*H Range of Dilutions	Average *p*H
I	5.60–6.19	5.96
II	5.99–6.70	6.14
III	5.09–5.34	5.20
IV	4.99–5.23	5.14
V	5.53–5.75	5.66
VI	5.30–5.51	5.43
VII	5.69–5.98	5.84
VIII	5.00–5.19	5.09
IX	1.64–2.53	2.05

TABLE I.—THE TEST COMPOUNDS

Compound I

(*p*-Nitro-phenyl copper tri-pyridine complex)

Compound II

(Phenyl-copper-octadecenonitrile tri-pyridine complex)

Compound III

(*p*-Tolyl copper tri-pyridine complex)

Compound IV

(*p*-Sulfonamido-phenyl copper tri-pyridine complex)

Compound V

(*p*-Nitro-phenyl-copper-propionitrile-tri-pyridine complex)

Compound VI

(*m*-Nitro-phenyl copper tri-pyridine complex)

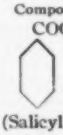
Compound VII

(*m*-Nitro-phenyl-copper-octanonitrile-tri-pyridine complex)

Compound VIII

(Phenyl-copper tri-pyridine complex)

Compound IX



(Salicylic acid)

THE TEST METHOD

Transfers of *Trichophyton gypseum*, *Epidermophyton interdigitale*, and *Trichophyton mentagrophytes* were made into tubes of liquid modified Czapek-Dox medium. After sufficient growth had appeared, Kolle flasks, containing solid modified Czapek-Dox medium, were inoculated with 1 cc. of the growth. The flasks were rotated to distribute the inoculum and then incubated in the dark for three to four days at between 22 to 25°.

After incubation, 20 cc. of sterile normal saline was added to each flask. The flask was gently shaken to dislodge the spores. This suspension was then filtered through two layers of sterile cheese cloth, into sterile 30-cc. centrifuge tubes. The spore suspensions were centrifuged and washed three times, then restored to the original volume with sterile normal saline. The spore count of this suspension was determined by the Decimal Dilution Method. The inoculum was standardized to contain two and a half million spores per cc.

At intervals of one minute, ten minutes, one hour, and twenty-four hours, 0.5 cc. of the inoculum was introduced into tubes containing 2 cc. of the compounds diluted to 1:250, 1:500, 1:1,000, 1:2,000 and 1:5,000, respectively. At the end of each contact period, a loopful of this mixture was transferred to slants and broth tubes of modified Czapek-Dox medium. These were incubated for three to five days at 22 to 25°. An additional five days' incubation was allowed for negative tubes to be confirmed.

In an attempt to rule out fungistasis, a loopful of the respective untreated spore suspension was added to all negative tubes. In every instance growth was observed after this treatment. This procedure suggested fungicidal activity of the compounds.

RESULTS

The results are recorded in Tables III and IV. Compounds VI, VII, and VIII appeared to have marked antimycotic activity against *T. gypseum;* compounds I, IV, and V exhibited a lesser degree of activity; and compounds II and III showed no activity. The control, salicylic acid, showed the greatest activity.

Against *E. interdigitale*, compounds II, III, IV, V, VI, VII, and VIII showed fungicidal activity in all dilutions after twenty-four hours' contact. Compound VI appeared to be more active than the others. It killed the spores in the 1:250 concentration after ten minutes contact. Salicylic acid showed greater activity against *E. interdigitale* than it showed against *T. gypseum*.

CONCLUSIONS

The activity of the organo-copper complexes tested was slight. Against *T. gypseum*, compound VII was the most active with compounds VI and VIII being nearly as effective. Compounds I, IV, and V were next in the order named; II and III exhibited no activity at all. Salicylic acid showed definite activity at all time intervals in dilutions of 1:250 and 1:500, in the one hour and twenty-four hours time intervals in dilution of 1:1,000 and only after twenty-four hours contact in the 1:2,000 concentration. The 1:5,000 dilution was not active.

TABLE III.—GROWTH AND INHIBITION OF THE TEST ORGANISM AFTER 24 HOURS' INCUBATION[a]

Compound	Dilution	X	Y	Z
I	A	−	−	+
	B	−	−	+
	C	−	+	+
	D	+	+	+
	E	+	+	+
II	A	+	−	+
	B	+	−	+
	C	+	−	+
	D	+	−	+
	E	+	+	+
III	A	+	−	+
	B	+	−	+
	C	+	−	+
	D	+	−	+
	E	+	+	+
IV	A	−	−	−
	B	−	−	+
	C	+	−	+
	D	+	−	+
	E	+	+	+
V	A	−	−	−
	B	−	−	−
	C	+	−	−
	D	+	−	−
	E	+	+	+

[a] After one, ten, and sixty minutes, all tubes showed growth.

KEY

Dilutions: A = 1:250, B = 1:500, C = 1:1,000, D = 1:2,000, E = 1:5,000

Test Organisms (Spore Count): X = *T. gypseum* (2,565,000 per cc.), Y = *E. interdigitale* (2,735,063 per cc.), Z = *T. mentagrophytes* (2,529,662 per cc.)

+ = Growth, − = No growth

Against *E. interdigitale*, activity appeared greater. Compound VI was most active, showing no growth at ten minutes, one hour and twenty-four hours contact with the 1:250 dilution. Next in order of activity were II, III, IV, V, VII, and VIII; all showing activity after twenty-four hours contact with the first four dilutions.

Introduction of a drop of the suspension of the respective organism on all negative tubes produced growth after three days' incubation. This suggested elimination of the possibility of inhibition due to carry-over of compounds. This appeared to establish total destruction of the spores.

Although the substances tested did not give promising enough results to warrant further testing or clinical use, many helpful factors were introduced into the method of testing antimycotic agents.

SUMMARY

The results of this work did not show the organo-copper complexes to be as active as had been expected. It appears, however, that the test method presented too severe demands on the antimycotic agents. This research introduces many desirable modifications: the inoculum contains only a known concentration of spores; contains no mycelium; and is free of metabolic waste products. Re-inoculation of all negative tubes with a loopful of the respective spore suspension, with growth subsequently occurring,

TABLE IV.—GROWTH AND INHIBITION OF THE TEST ORGANISM AFTER VARYING PERIODS OF INCUBATION

Compound	Dilution	1 Minute			10 Minutes			1 Hour			24 Hours		
		X	Y	Z	X	Y	Z	X	Y	Z	X	Y	Z
VI	A	+	+	+	+	−	+	+	−	+	−	−	+
	B	+	+	+	+	+	+	+	+	+	−	−	+
	C	+	+	+	+	+	+	+	+	+	−	−	+
	D	+	+	+	+	+	+	+	+	+	−	−	+
	E	+	+	+	+	+	+	+	+	+	+	+	+
VII	A	+	+	−	+	+	−	+	+	−	−	−	−
	B	+	+	+	+	+	+	+	+	+	−	−	−
	C	+	+	+	+	+	+	+	+	+	−	−	+
	D	+	+	+	+	+	+	+	+	+	−	−	+
	E	+	+	+	+	+	+	+	+	+	+	−	+
VIII	A	+	+	+	+	+	+	+	+	+	−	−	−
	B	+	+	+	+	+	+	+	+	+	−	−	−
	C	+	+	+	+	+	+	+	+	+	−	−	−
	D	+	+	+	+	+	+	+	+	+	−	−	−
	E	+	+	+	+	+	+	+	+	+	+	−	+
IX	A	−	−	−	−	−	−	−	−	−	−	−	−
	B	−	−	−	−	−	−	−	−	−	−	−	−
	C	+	−	+	+	−	+	−	−	−	−	−	−
	D	+	−	+	+	−	+	+	−	−	−	−	−
	E	+	+	+	+	+	+	+	+	−	+	−	−

KEY

Dilutions:
A = 1:250
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X = *T. gypseum* (2,565,000 per cc.)
Y = *E. interdigitale* (2,735,063 per cc.)
Z = *T. mentagrophytes* (2,529,662 per cc.)
+ = Growth
− = No growth

suggests the probability of fungistasis being ruled out. The medium used here allows satisfactory growth in about one-half the time required when using other media.

The hydrogen-ion concentration of the dilutions of the compounds varied so little that no significant correlation could be made between fungicidal activity and *p*H. Salicylic acid, however, presents the greatest activity and at a *p*H range of from 1.6 to 2.5. Although this suggests an acid *p*H for activity, there was insufficient evidence here to propose this. Salicylic acid acted directly upon the fungus. This conclusion challenges the belief of some investigators (3) that its action in the treatment of dermatophytosis was due only to exfoliation of epidermis.

The relative resistance of the organisms used points to *M. roseum* as the test organism of choice, as it appears the most resistant. However, *M. roseum* proved to be a saprophytic *Fusarium*, *T. mentagrophytes* (27). The use of pathogenic fungi is more desirable. Emmons (28) suggests that *E. interdigitale*, U.S.P.H.S. strain No. 640, be employed because of its uniform resistance.

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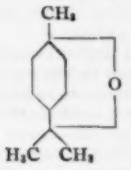
The Spectrophotometric Determination of Cineole (Eucalyptol)*

By ERIC W. MARTIN and JOSEPH W. E. HARRISSON

A procedure is presented for the determination of small quantities of eucalyptol in pharmaceutical preparations. This analytical method yields satisfactory results when various expectorants and antiseptics, such as menthol, benzyl alcohol, thymol, and camphor are present.

CINEOLE was first officially recognized under the title Eucalyptol in the U. S. Pharmacopeia of 1890. It is classified organically as an inner ether, or more specifically as the anhydride of 1,8-menthanediol and has the following formula:



Although important pharmaceutically because of its germicidal and expectorant properties (1), a satisfactory method for its rapid and accurate determination in medicinal preparations has never been described in the literature. The official compendia (National Formulary and United States Pharmacopeia) have provided qualitative tests for establishing minimum requirements for purity, but they have never included an assay procedure.

Several methods are used for its estimation in eucalyptus and other oils but none of these is a specific chemical assay. The most useful quantitative procedures reported to date depend on physical determinations such as the melting point of the molecular compound (cresineol) formed with *o*-cresol (2). This method was adopted by the British Pharmacopœia of 1932 for eucalyptus oil instead of the inaccurate H_3PO_4 method (3). Kleber and Rechenberg have also determined cineole in eucalyptus oil by means of freezing points at low temperatures (4). This is the basis for the U. S. Pharmacopeia congealing point requirement of a minimum of $-15.4°$, which indicates not less than 70 per cent of cineole.

Within the last twenty-five years a large number of color tests have appeared in the literature for the detection of eucalyptol (5,6). Most of the colors are developed by reacting cineole with an aldehyde or phenol in the presence of sulfuric acid. The hue and intensity of the colors produced vary with the reagents employed. These have included furfural, anisaldehyde, salicylaldehyde, vanillin, cinnamaldehyde, piperonal, α-naphthol, and β-napthol. The colors have been described by vague terminology and have been found, during the present investigation, to be unstable. The blue color produced with ammonium molybdate has also been used as a color identity test (7). Wasicky (8) has reviewed the methods for detecting cineole.

Spectrophotometric examination of the colors produced with various reagents led to the development of the simple and rapid assay procedure for cineole described below.

EXPERIMENTAL

Materials

The absorption spectra and the absorbancies reported in this paper were determined in 1-cm., calibrated, matched silica cells with a Model DU Quartz Spectrophotometer. All absorbancies reported are corrected. All solvents used were of reagent grade.

p-Dimethylaminobenzaldehyde Reagent (DAB).—This reagent is prepared by cautiously adding 640 ml. of concentrated sulfuric acid to 400 ml. of water. The mixture is continually cooled. In the cooled acid solution (75% H_2SO_4) is dissolved sufficient p-dimethylaminobenzaldehyde to make a 0.5% w/v solution.

Cineole.—Merck's Eucalyptol U. S. P.

Development of Method

The spectra of the colors proposed as identity tests for cineole were examined for possible usefulness as a basis for its colorimetric determination. It was found that p-dimethylaminobenzaldehyde in the presence of sulfuric acid produced a characteristic and reproducible red color with cineole at ordinary temperatures when precautions were taken to control time, concentrations of reactants, and moisture.

The absorption spectra obtained during the reaction with the reagent (DAB) are shown in Fig. 1. During the first few minutes of the reaction, two peaks rapidly develop at λ400 and λ555, with a minimum at about λ450. Curve *C* in Fig. 1 shows the spectrum of the red color about one minute after mixing 200 μg. of cineole, dissolved in 2 ml. of anhydrous methanol, with sufficient DAB

* Received May 5, 1950, from the Research Laboratories of LaWall and Harrisson, Philadelphia, Pa.
Presented to the Scientific Section, A.Ph.A., Atlantic City meeting, May, 1950.

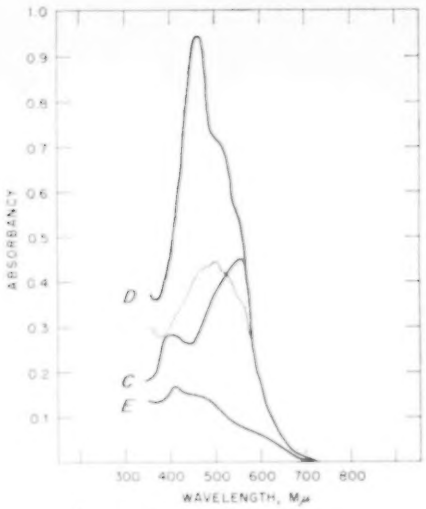


Fig. 1.—Spectra of cineole colors.

to make 25 ml. These peaks are fully formed in about six minutes. The rate of absorbancy development at λ555 is shown in Fig. 2. After approximately six minutes there is little or no further development of color when pure solutions of cineole are used. Curve *A* in Fig. 2 indicates the intensity of the absorption maximum at λ555 at various time intervals after 250 μg. of cineole dissolved in 2 ml. of methanol is mixed with sufficient DAB to make 25 ml. Curve *B* in Fig. 2 indicates the rate of color formation with 100 μg. of cineole.

After a period of about three hours the minimum becomes inverted and a region of maximum absorbancy appears at λ500. See the dotted curve in Fig. 1, which is the spectrum produced from 250 μg. of cineole in 2 ml. of methanol diluted to 25 ml. with DAB. During the three-hour period the color changes from a brilliant red to reddish orange. If the reaction mixture is diluted with 14 *N* sulfuric acid (a more dilute acid) the inversion of the maximum occurs more rapidly and a much more intense absorption occurs at λ480. Curve *D* in Fig. 1 is the spectrum produced when 1 ml. of 1 mM cineole in anhydrous methanol is mixed with 5 ml. of DAB, allowed to stand for thirty minutes, and then diluted with 14 *N* sulfuric acid to 50 ml. After eighteen hours considerable fading takes place, all peaks are diminished to the positions shown by Curve *E* in Fig. 1, and the color becomes yellow.

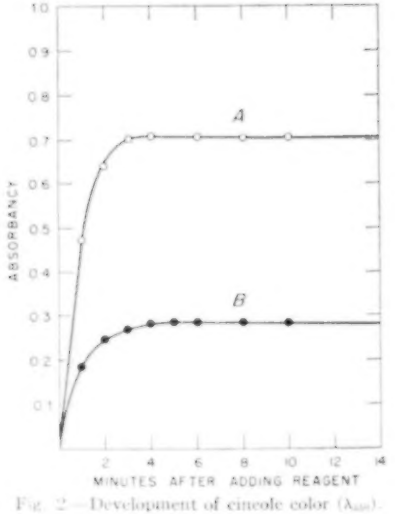


Fig. 2.—Development of cineole color (λ_{555}).

It was found that the peak at λ555 could be stabilized after complete development at about six minutes by using a ratio of 23 parts of DAB to 2 parts of an anhydrous methanol solution of cineole. Too much DAB caused the reaction to be unduly prolonged, and too little caused very rapid development of color followed by rapid fading. No further dilution is necessary when a concentration of 50 to 250 μg. per ml. of methanol is used. If the developed color is diluted with solvents such as methanol and water, rapid fading occurs, but dilution can be made with 75% sulfuric acid.

At the present time the structure of the product or products formed by the reaction between cineole and *p*-dimethylaminobenzaldehyde is unknown, but undoubtedly a condensation product is formed between these two compounds because either one alone in contact with sulfuric acid does not produce an absorption spectrum even after prolonged standing.

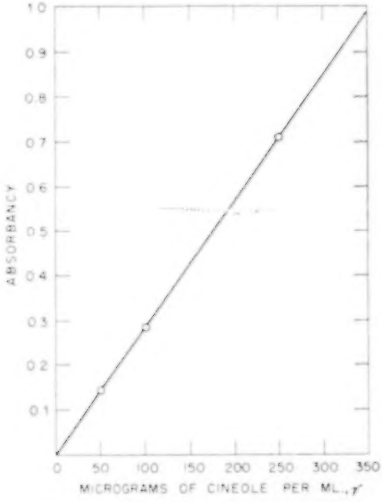


Fig. 3.—Standard curve for cineole.

The standard curve shown in Fig. 3 was prepared as follows. Accurately weighed quantities of cineole were dissolved in anhydrous methanol and 2-ml. aliquots of these solutions were diluted with sufficient DAB to make 25 ml. of solution and thoroughly mixed. The absorbancies of the brilliant red color at λ555 were determined in a spectrophotometer in exactly six minutes, the period re-

quired for maximum color development. The absorbancies thus obtained were plotted against the corresponding concentrations.

Assay Procedure

The pharmaceutical preparation being assayed must be treated in such a manner that all the eucalyptol is brought into solution in anhydrous methanol in a concentration of 50 to 250 μg./ml. The method of treatment will depend on the type of preparation.

Pipette a 2-ml. aliquot of the solution into a 25-ml. glass-stoppered flask, make up to volume with DAB, and mix thoroughly by repeatedly inverting the flask. Read the absorbancy at λ555 in a suitable spectrophotometer six minutes after the reagent is added. The absorbancy so obtained may be converted either to micrograms of cineole per ml. by referring to the standard curve (see Fig. 3) or to concentration expressed in per cent by dividing the absorbancy by the $E^{1\%}_{1\ cm}$ value of 28.40.

DISCUSSION

The results of analyses as applied to known solutions of cineole are remarkably accurate. Table I indicates the average per cent deviation from theory experienced with this method is approximately ±0.5%.

Solutions 1, 2, and 3 were pure solutions of cineole in anhydrous methanol. In addition to the cineole, solution 4 contained 0.150% benzyl alcohol, 0.05% menthol, and 0.038% camphor; solution 5 contained 300 γ/ml. of benzyl alcohol, 500 γ/ml. of menthol, and 76 γ/ml. of camphor; and solution 6 contained 150 γ/ml. of benzyl alcohol, 250 γ/ml. of menthol, 38 γ/ml. of camphor and 100 γ/ml. of thymol.

It was found that the presence of thymol appears to hinder color development slightly, and a somewhat longer time (ten minutes) must be allowed for maximum color development. Further experimentation is being undertaken to increase the applications of the method in the presence of interfering substances. A study of the analyses shown in Table I reveals the fact that no interference is experienced with menthol, benzyl alcohol, and camphor, and practically none with thymol.

TABLE I.—ASSAY OF CINEOLE SOLUTIONS

Solution No.	Concentration of Cineole—Theoretical	Concentration of Cineole—Found	Deviation, %
1	100 γ/ml.	100 γ/ml.	0
2	192 γ/ml.	192 γ/ml.	0
3	50 γ/ml.	50.2 γ/ml.	0.4
4	0.0385%	0.03 875%	0.65
5	125 γ/ml.	124.4 γ/ml.	0.48
6	62.5 γ/ml.	62 γ/ml.	0.8

The method is remarkably sensitive. It is possible to determine with considerable accuracy concentrations of cineole of the order of 50 μg./ml.

It is suggested that the official compendia include at least a purity and identity test utilizing the above reaction for cineole.

A procedure, similar to that described in this paper but utilizing other light frequencies, has been developed for the analysis of related terpenes, and will be published soon.

SUMMARY

1. A spectrophotometric method for assaying cineole (eucalyptol) in the presence of menthol, thymol, benzyl alcohol, camphor, and other antiseptics and expectorants is described.

2. The procedure is simple, rapid, and accurate. Eucalpytol, in methanol solutions containing the above substances, was determined with an average accuracy of ±0.5 per cent.

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A Colorimetric Method for the Estimation of Some Tropine Alkaloids*

By FRANCES DURICK, J. STANTON KING, Jr., PALMER A. WARE, and GEORG CRONHEIM

The acid dye method for the estimation of certain alkaloids and other base compounds has been extended to atropine and other tropine alkaloids. This procedure is based on the observation that certain acidic dyes combine with many alkaloids to form saltlike compounds which are characterized by their solubility in organic solvents. They can thus be separated and the dye component can be estimated colorimetrically. The procedure has been adapted to the determination of atropine and hyoscyamine with the help of bromocresol purple. The method permits the quantitative estimation of these alkaloids in amounts ranging from 0.1 to 2.0 mg. per sample. Applications of the method to tinctures and extracts of belladonna and hyoscyamus will be discussed.

THE U. S. P. method for the assay of preparations containing tropine alkaloids is not very practical for their rapid and accurate estimation in small quantities. The purpose of this study was to apply the acidic-dye method (1–7), which is especially useful in the estimation of small amounts of materials, to the assay of these alkaloids.

The procedure is based on the observation that a number of alkaloids and other organic bases form, with some acidic dyes, saltlike addition compounds which are characterized by their solubility in certain organic solvents. After subsequent decomposition with alkali, the dye can be estimated colorimetrically. The procedure used in this study follows closely that for methadone (5) with bromocresol purple as the acid component and benzene as the organic solvent, comparison having indicated these reagents to be relatively satisfactory.

Experiments show that the method works quite well with atropine and hyoscyamine, while scopolamine (hyoscine) acts less satisfactorily. The procedure is also applicable, especially for comparative purposes such as time-stability tests, uniformity tests, etc., to belladonna and hyoscyamus in their usual preparations (extract, tincture, etc.)

EXPERIMENTAL

Reagents.—Concentrated phosphate buffer of *p*H 5.3: Dissolve 19.0 Gm. of monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) and 1.0 Gm. of anhydrous dibasic sodium phosphate (Na_2HPO_4) in distilled water and dilute to 500 ml.

Dye solution: Dissolve 0.200 Gm. of bromocresol purple in 15 ml. of distilled water and 3.2 ml. of 0.1 *N* sodium hydroxide and dilute to 250 ml.

Benzene, C. P.

Chloroform, C. P.

Sodium hydroxide, 0.05 *N*.

Procedure.—Five milliliters of the buffer solution and 5 ml. of the dye solution are measured into a separatory funnel and 10 ml. of the sample, containing 0.1 to 2.0 mg. of alkaloid, is added and mixed. Fifty milliliters of benzene is added and the mixture shaken for three minutes. After separation, the benzene layer is removed and the extraction repeated with a second 50-ml. portion of benzene. The two benzene extracts are combined and water globules removed by centrifugation. An 80-ml. aliquot is transferred to another separatory funnel and extracted with two 10-ml. portions of 0.05 *N* sodium hydroxide. The combined sodium hydroxide layers are diluted to exactly 25 ml. with 0.05 *N* sodium hydroxide. Filtration through cotton may be necessary to clarify the solution. The transmittancy is measured in a colorimeter[1] and compared with a standard curve prepared by the same procedure. The same batch of dye must be used in all experiments, because of purity variations. The color produced is heat-stable and gives a constant reading for at least two hours. The sensitivity of the method may be increased by repeating the extraction, by the use of cuvettes having a longer light path, and/or by reducing the volume of sodium hydroxide solution used in the final step.

Contrasted with the methadone addition product, the alkaloid-dye addition products of those tropine alkaloids studied are not very soluble in benzene. It is important that the volumes of the aqueous and the nonaqueous phases be measured correctly and always kept in the same proportion in any given series of experiments.

The preceding method refers to pure alkaloidal salts, which contain no interfering materials. Botanical drugs may be similarly assayed. If most of the chromogens are removed by centrifugation, i.e., if the resulting supernatant is considerably less colored than the starting material, most of the interference is thereby removed. A sample of the resulting supernatant of appropriate size is evaporated to a syrup on the steam bath. This procedure renders insoluble other interfering materials. The residue is transferred with several washings of 0.01 *N*

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[1] We used 19-mm. cuvettes in a Coleman spectrophotometer, model 11, set at 580 mμ, with secondary filter PC-4.

H_2SO_4 to a retentive filter paper. Occasionally, the residue on evaporation is completely soluble in the acid wash, and thus passes through the filter paper. Since the procedure fails to remove all interfering material, the preparation must be taken through the longer U. S. P. procedure (9). The filtrate is adjusted to pH 5.2 to 5.5 and to a volume such that 10 ml. contains about 1 mg. of alkaloid. The assay is then completed as described. Hyoscyamus should be read on the hyoscyamine curve and belladonna on the atropine curve, since these are their respective major active constituents (8). When this purification can be used, the time required for an assay is materially decreased.

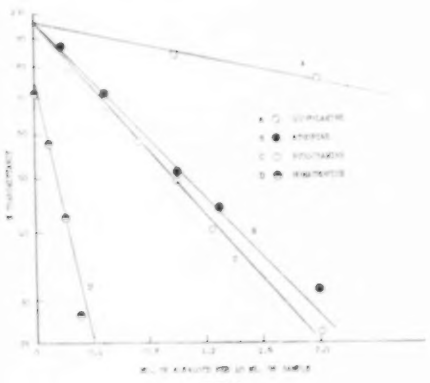
RESULTS

Typical results of a number of individual determinations with salts of atropine, hyoscyamine, and scopolamine are presented in Fig. 1. It is apparent that within proper limits the results follow Beer's law and are quite reproducible. Under the conditions used, where the sample contained from 0.1 to 2.0 mg. of the alkaloid, the error did not exceed ±5%. Alterations in the sensitivity of the reaction, as reflected by shifts in the position of the standard curves, were encountered on different days, although the relative positions of the curves remained the same. The cause of this shifting has not been found; although alterations of shaking time, reaction temperature, batches of dye, buffer, benzene, and alkaloidal salts were made, none of these were found to alter the curves very significantly. Often the position of the curves remained the same for weeks. Such shifts are of course made unimportant by preparing the standard curve at the same time as the sample, and the standard curve may be easily checked by determining whether a given point determined on subsequent days coincides with the same point on the original curve. The blanks do not change significantly in value.

In the case of scopolamine, the results with the pure alkaloidal salt differ in that the color obtained is much weaker than that from equivalent quantities of the other two alkaloids; scopolamine as the free base gives no better results. The explanation must be found in variations either in the affinity of the base for the dye acid or in the solubility of the resulting salt in benzene. Use of chloroform instead of benzene increases the sensitivity but also increases the blank reading. The figure also shows the results obtained with homatropine methyl bromide, which gave practically no color using benzene, but gave the indicated results with chloroform as the organic phase.

A stoichiometric calculation indicates that for both atropine and hyoscyamine the final dye solution contains only about $^1/_{20}$ mole equivalent of the alkaloid per mole equivalent of the dye, while for scopolamine the proportion is still smaller. This is in contrast to the results with methadone where a dye-alkaloid ratio of 1:2 was found (5) corresponding to the dibasic character of bromocresol purple. Since it is rather improbable that sufficiently reproducible results may be obtained under conditions where the affinity of the organic base for the acidic dye is low, it appears more likely that the dye-base compound is not very soluble in benzene and is, therefore, only partially extracted. Evidently the latter assumption is correct, since considerable additional amounts of the dye-atropine compound may be extracted from the test solution with successive portions of benzene (Table I). It is believed that differences in the solubilities and the resulting variations in the distribution coefficient account for the observed differences between the three studied tropine alkaloids.

The selection of the proper pH for the formation and extraction of the alkaloid-dye compound is very important. Figure 2 shows the per cent transmittancy (logarithmic scale) at various pH values with 1 mg. of atropine and also the corresponding blank readings. It indicates that the best results are obtained using a pH of about 5.5. At this point the difference between the blank and the test solution is at a maximum and the blank reading itself is at a minimum.



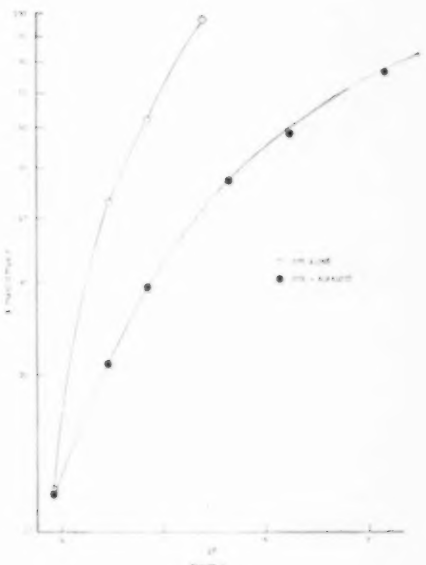


TABLE I

Extraction	Transmittancy,[a] %	Extraction	Transmittancy,[a] %
1st	16.5	9th	48.0
2nd	18.5	10th	51.5
3rd	22.5	11th	59.5
4th	26.5	12th	58.5
5th	30.0	13th	70.0
6th	34.5	14th	64.0
7th	42.0	15th	64.5
8th	41.5	Blank	85.0

[a] 40-mm. light path.

In extending this method from pure alkaloids or their salts to U. S. P. preparations of belladonna and of hyoscyamus, a preliminary purification by the official U. S. P. procedure (9), to remove chlorophyll and other extraneous material, is often necessary. The assay procedure is then the same as for pure alkaloids. Attempts to simplify this purification were successful only in cases that were arbitrarily described previously. The relative interference with this short method was too great to permit its use with tincture of hyoscyamus, U. S. P. With such preparations attempts were made to adsorb selectively the alkaloids and leave behind the interfering impurities, which gave consistently high results of the indicated magnitude (Table II). Activated charcoal was found to remove both the alkaloids and the interfering impurities; Superfiltrol removed the alkaloids and some of the interfering impurities, as determined by filtering and assaying the filtrate.

TABLE II

Preparation	U. S. P. Assay, Mg. %	With U. S. P. Purification, Mg. %	With Short Purification, Mg. %
Fldext. belladonna	300		304
Fldext. belladonna	308		286
Fldext. belladonna	463		424
Fldext. hyoscyamus	37	[a]	35.2
Fldext. hyoscyamus	39	44	107 108 91.2
Fldext. hyoscyamus	40		48.8
Fldext. hyoscyamus		21.6	58.0
Tinct. belladonna	32.8	34.4	38.8
Tinct. belladonna	32.1		40.0
Tinct. hyoscyamus	3.53	4.76 3.53 3.79 4.41	
Tinct. hyoscyamus	4.10		10.1
Tinct. hyoscyamus	3.82	4.76	10.0

[a] Not suitable for short determination.

Hydrolysis of the alkaloids results in a blank reading. Choline and asparagine, which are also present in some of these preparations, do not give interference under these conditions, nor does chlorophyll, in the amounts normally carried over. Table II illustrates some typical results for the intercomparison of the U. S. P. assay (10), the U. S. P. preliminary purification (9) with this method, and the purification technique described above with this method. Results are included for some preparations with which interference was encountered. Samples were from different batches and from three manufacturers.

In connection with the present investigation a short study was made concerning the effect of two filter aids used in laboratory and manufacturing procedures on the alkaloids of belladonna and hyoscyamus tinctures. It was found that Superfiltrol[2] removes the alkaloids, while Celite[3] is without noticeable effect.

SUMMARY

1. A method is described for the assay of 0.1 to 2.0 mg. samples of salts of atropine, hyoscyamine, scopolamine, or homatropine involving the formation of an addition product with bromocresol purple, its extraction into benzene or chloroform, and the decomposition of the product in alkaline solution. The color of the dye component is then estimated colorimetrically.

2. The method may be applied to U. S. P. preparations containing these alkaloids.

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(10) *Ibid.*, pp. 67, 263.

[2] Thiamine grade, obtainable from the Filtrol Corp., 634 Spring St., Los Angeles 14, Calif.

[3] Medium, hi-flo, obtainable from Johns-Manville Products, New York 16, N. Y.

The Quantitative Determination of Pregnenolone and Pregnenolone Acetate by Infrared Spectrophotometry*

By G. PAPINEAU-COUTURE and R. A. BURLEY†

An infrared spectrophotometric method is described for the quantitative determination of pregnenolone and its acetate in pharmaceutical preparations. Optical density measurements are made at several intense absorption peaks, and the concentration is calculated independently from each measurement. The agreement between the different measurements is an indication of the presence or absence of impurities.

BECAUSE of the present interest in pregnenolone and its derivatives for the treatment of rheumatoid arthritis, it has become necessary to develop a quantitative analytical procedure for its determination in pharmaceutical preparations.

Pregnenolone possesses no specific chemical properties other than those common to alcohols and ketones. The quantitative precipitation of the 2,4-dinitrophenylhydrazone has been proposed as an analytical method. Such a procedure is applicable only to preparations containing pure pregnenolone or its acetate since any ketonic impurity present would also be precipitated.

The necessity for a more specific method has led us to develop an infrared spectrophotometric procedure.

In any spectrophotometric analytical method, three main considerations govern the choice of absorption bands: (*a*) they should have sufficient intensity to be accurately measurable at as low a concentration as possible; (*b*) they should follow Beer's law; and (*c*) they should be specific of the compound.

The absorption spectrum of pregnenolone acetate in carbon disulfide (Fig. 1) has four very strong absorption bands, all of which satisfy the first two conditions. Their optical density can be measured accurately in a 1 mm. cell at a concentration of 1 to 2 mg./ml., and, as can be seen from Fig. 2, they obey Beer's law quite closely. However, none of these bands is in itself characteristic of the compound.

Furchgott, Rosenkrantz and Shorr (1) and Jones, *et al.* (2) have shown that each of these absorption bands can be attributed to the vibration of a definite bond. These structural assignments are indicated in Fig. 1. Since all the bonds involved belong to functional groups, none of which is peculiar to pregnenolone acetate, the measurement of a single band could not be considered any more specific than the precipitation of the dinitrophenylhydrazone. It was, however, expected that the relative intensities of the four bands would be characteristic of the compound.

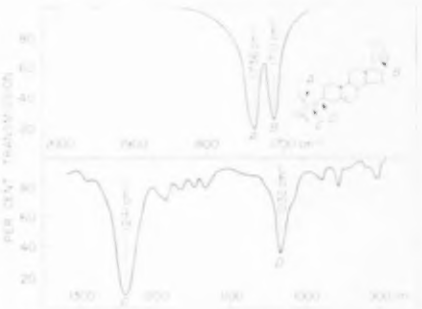

Fig. 1.—Pregnenolone acetate per cent transmission graph. (CS_2 solution, 5 mg./ml., 1-mm. cell, NaCl prism.)

The influence of interfering contaminants on the relative intensities of these bands was therefore studied by measuring the optical densities of mixtures consisting of pregnenolone acetate and a steroid having at least one common group. In these mixtures, the proportions of contaminant and pregnenolone acetate were varied, keeping the total weight of sample as constant as possible. With pregnenolone as the impurity (Table I), the intensity of the two bands due to the acetate group (1736, 1241 $cm.^{-1}$) and of that due to the C_3 acetoxy bond (1032 $cm.^{-1}$) decreases rapidly with decreasing amount of pregnenolone acetate, while the 1710 $cm.^{-1}$ band due to the 20-keto grouping remains essentially constant. Hence, in this case, measurement of the latter band alone would obviously not be a suitable analytical procedure. Table II shows a similar behavior when dehydroisoandrosterone acetate, having the common Δ^5, 3*β*-acetate structure, was the contaminant. In this case, only the 1710 $cm.^{-1}$ band parallels the

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† The interest taken in this work by Dr. Gordon A. Grant, Director of Research, is gratefully acknowledged by the authors.

TABLE I.—OPTICAL DENSITIES OF MIXTURES OF PREGNENOLONE ACETATE (A) AND PREGNENOLONE (B)

—Conc., Mg./Ml.— A	B	Total	1736 $Cm.^{-1}$	1710 $Cm.^{-1}$	1241 $Cm.^{-1}$	1032 $Cm.^{-1}$
1.56	0.0	1.56	0.260	0.187	0.409	0.155
			0.256	0.187	0.403	0.155
1.04	0.50	1.54	0.184	0.187	0.284	0.114
			0.184	0.187	0.280	0.114
0.52	1.00	1.52	0.094	0.181	0.152	0.071
			0.097	0.184	0.152	0.071
0.0	1.50	1.50	0.007	0.181	0.022	0.027
			0.007	0.174	0.020	0.027

TABLE II.—OPTICAL DENSITIES OF MIXTURES OF PREGNENOLONE ACETATE (A) AND DEHYDROISOANDROSTERONE ACETATE (B)

—Conc., Mg./Ml.— A	B	Total	1736 $Cm.^{-1}$	1710 $Cm.^{-1}$	1241 $Cm.^{-1}$	1032 $Cm.^{-1}$
1.56	0.0	1.56	0.272	0.215	0.420	0.161
1.04	0.47	1.51	0.292	0.187	0.403	0.155
0.52	1.02	1.54	0.280	0.071	0.357	0.137
0.0	1.57	1.57	0.332	0.015	0.372	0.143

concentration of pregnenolone acetate, while the variations in optical density at the other frequencies do not follow the changes in composition of the mixture.

No single band is, therefore, characteristic of pregnenolone acetate. However, the particular combination of these four bands is characteristic of this compound because the chance that another compound will absorb at all these frequencies with the same relative intensity is extremely remote. Hence, it appears that the measurement of the optical density of pregnenolone acetate at the four main frequencies constitutes a specific analytical tool.

A method based on the same principle has been developed for free pregnenolone.

Figure 3 shows the absorption spectrum of a carbon disulfide solution of pregnenolone. A comparison of this spectrum with that of the acetate shows that the two bands due to the acetate group (1736 and 1241 $cm.^{-1}$) have disappeared, the 1032 $cm.^{-1}$ band is displaced to higher frequency at 1047 $cm.^{-1}$ (probably due to the substitution of an H for the CH_3—C=O radical) whereas the 1708 $cm.^{-1}$ band is still present. Due to the low solubility of pregnenolone in carbon disulfide, the spectrum was obtained using a 3-mm. cell. The three bands chosen for the quantitative measurements are indicated in Fig. 3 and their agreement with Beer's law is shown in Fig. 4. The 953 $cm.^{-1}$ band is considerably weaker than the other two, and since it was desired to make all measurements on one solution, a 1-mm. cell was used for the stronger bands, whereas the weak one was measured in a 6-mm. cell.

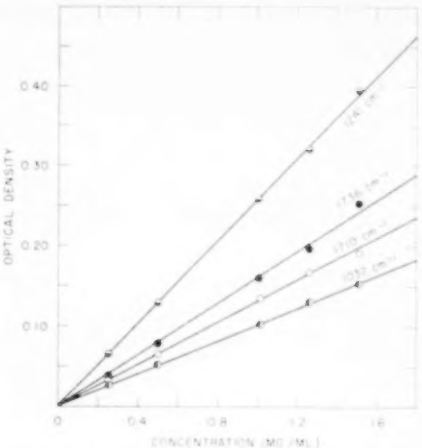

Fig. 2.—Beer's law plot for pregnenolone acetate. (Solvent: CS_2; cell thickness: 1 mm.).

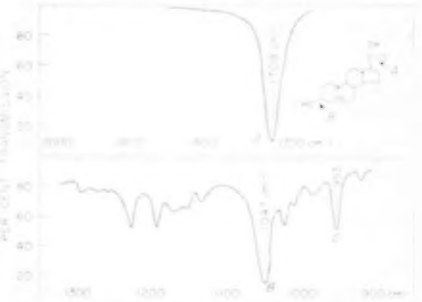

Fig. 3.—Pregnenolone per cent transmission graph. (Saturated CS_2 solution, 3-mm. cell, NaCl prism.)

EXPERIMENTAL

Instrument.—All the measurements reported here were made with a Perkin-Elmer model 12C recording infrared spectrophotometer equipped with a sodium chloride prism. The cells consisted of 1- and 6-mm. brass spacers fitted with sodium chloride windows.

Standards.—The pure steroids were obtained from Syntex S.A. Pregnenolone was recrystallized from analytical reagent methanol, m. p. 189–191°. Pregnenolone acetate was recrystallized from aqueous acetone, m. p. 150–152°.

DETERMINATION OF PREGNENOLONE ACETATE

Preparation of Samples

Standard and Bulk Material.—About 250 mg. is weighed accurately into a 50-ml. volumetric flask and the flask filled to the mark with acetone. Three-milliliter aliquots of the acetone solution are pipetted

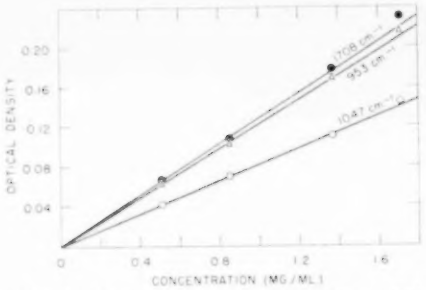

Fig. 4.—Beer's law plot for pregnenolone. (Solvent: CS_2; cell thickness, 1 mm.: ●, ○, 6 mm.: ▲, △.)

into 25-ml. glass-stoppered Erlenmeyer flasks, and evaporated to dryness by heating over a steam bath under a stream of nitrogen. The residue is dissolved in 10 ml. CS_2 just before measuring the optical densities. All determinations are done in triplicate.

Tablets.—Ten tablets (claimed to contain 100 mg. per tablet) are weighed accurately and ground in a mortar. About one-half of the resulting powder is weighed accurately, placed in a sintered glass funnel and extracted repeatedly by slurrying the powder in acetone, then filtering with suction. The extracts are collected in a 100-ml. volumetric flask and the volume is made up with acetone. Aliquots of 3 ml. are treated as above.

Optical Density Measurements

The approximate wave length drum setting corresponding to each of the four bands marked *A*, *B*, *C*, and *D* in Fig. 1 is found from a qualitative record. With a solution of the standard in the 1-mm. cell, the exact settings are determined by manual operation.

With the 1-mm. cell filled with pure CS_2, the slit width necessary to give 100% pen deflection on the recorder is determined for each of the above settings.

The same cell is filled with the standard solution and the pen deflection is noted for each band using the corresponding slit width predetermined with the pure solvent. The standard optical density is then calculated from the equation:

$$K_{0.1} = D/C \qquad (1)$$

where

$K_{0.1}$ = optical density of a 0.1% solution
D = actual optical density of the solution
C = concentration of the solution (mg./ml.)

A $K_{0.1}$ value is thus obtained for each of the four bands.

The optical density of the unknown is measured using the same procedure and the concentration is calculated at each wave length by the formula:

$$C = D/K_{0.1} \qquad (2)$$

where

C, D = concentration and optical density of the unknown, respectively
$K_{0.1}$ = as defined in Eq. 1

A typical analysis is given in Table III. The agreement of the concentration values obtained from the four bands is typical of the method. In this laboratory, the average deviation has never been greater than 0.02 mg./ml. for a concentration of 1.5 mg./ml.

TABLE III.—TYPICAL ANALYSIS OF PREGNENOLONE ACETATE

Frequency:	Optical Density Values 1736 Cm.⁻¹	1710 Cm.⁻¹	1241 Cm.⁻¹	1032 Cm.⁻¹
Standard (1.50 mg./ml.)	0.248	0.194	0.409	0.155
	0.244	0.194	0.414	0.155
	0.244	0.194	0.403	0.155
Average	0.245	0.194	0.409	0.155
$K_{0.1}$	0.163	0.129	0.272	0.103
Unknown	0.244	0.194	0.398	0.152
	0.240	0.187	0.398	0.155
	0.240	0.187	0.398	0.152
Average	0.241	0.189	0.398	0.153
Concentration	1.48	1.47	1.46	1.49

Average concentration: 1.48 ± 0.01 mg./ml.

DETERMINATION OF PREGNENOLONE

Preparation of Samples

Standard and Bulk Material.—This is done as described for pregnenolone acetate except that analytical reagent methanol is used instead of acetone in the preparation of the original solution. (Crystals deposited from methanol are more easily soluble in carbon disulfide.)

Aqueous Suspensions.—Vials are claimed to contain 1 Gm. in 10 ml. water. The contents of five vials are filtered through sintered glass funnels, the solids washed with water, dried, and weighed.

A 250-mg. aliquot of the pooled solids is weighed and dissolved in 50 ml. methanol. Aliquots of 3 ml. are evaporated to dryness and dissolved in 10 ml. CS_2.

Optical Density Measurements

The three bands marked *A*, *B*, and *C* in Fig. 3 are measured as described for pregnenolone acetate. As mentioned previously, the 1708 and 1047 cm.⁻¹ bands are measured in a 1-mm. cell but the third band is measured in a 6-mm. cell, using the same solution. This, of course, applies to the blank, the standard, and the unknown.

TABLE IV.—TYPICAL ANALYSIS OF PREGNENOLONE

Frequency:	Optical Density Values 1708 Cm.⁻¹	1047 Cm.⁻¹	935 Cm.⁻¹
Standard (1.45 mg./ml.)	0.181	0.125	0.187
	0.187	0.125	0.194
	0.194	0.125	0.194
Average	0.187	0.125	0.192
$K_{0.1}$	0.129	0.086	0.132
Unknown	0.181	0.122	0.191
	0.181	0.116	0.184
	0.187	0.122	0.187
Average	0.183	0.120	0.187
Concentration	1.42	1.39	1.42

Average concentration: 1.41 ± 0.01 mg./ml.

Typical results are given in Table IV. It will be seen that the accuracy of the results is the same as for the acetate.

SUMMARY AND CONCLUSIONS

1. An analytical method has been developed for the quantitative determination of pregnenolone and its acetate.

2. Since the spectra of the compounds contain no strong specific absorption bands, the method is based on the measurement of several nonspecific bands.

3. It is claimed that the particular combination of optical intensities at the chosen wave lengths is a characteristic property of each compound.

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The Determination of Resorcinol in Pastes and Ointments*

By LLEWELLYN H. WELSH

The official method for the determination of resorcinol in N. F. pastes of resorcinol presents mechanical difficulties. A method of analysis is described which eliminates these difficulties and which is applicable to certain nonofficial preparations to which the official method of analysis is not suited.

THE National Formulary (1) contains monographs for two pharmaceutical preparations composed of resorcinol, zinc oxide, starch, and light liquid petrolatum. These preparations are strong resorcinol paste and mild resorcinol paste which contain, respectively, 20 per cent and 10 per cent resorcinol. The monographs in the sixth and subsequent editions of the N. F. include a procedure, based on the report of Baker (2), for the determination of resorcinol. For the extraction of this ingredient from the paste, the procedure depends on shaking a sample with portions of hot water. The aqueous extracts are then separated from the other components by filtration, and resorcinol is determined by brominating an aliquot of the filtrate.

In the hands of the author, the extraction of resorcinol from strong resorcinol paste according to the official method proved to be a tedious process. The filter tended to become clogged by the pasty phase to such an extent that filtration usually required several hours. In some instances filtration proceeded so slowly that it was expedient to abandon the determination and begin another. That the N. F. method is not suitable for certain other preparations qualitatively similar to, but of considerably thicker consistency than, strong resorcinol paste was made evident by an attempt to apply the official method to an ointment having the composition of that listed in Table I. When shaken with hot water, a sample of the ointment resisted disintegration to a large extent, filtration was slow, and bromination of the filtrate showed that little more than half (53 per cent) of the resorcinol had been extracted.

In addition to presenting mechanical difficulties related to the extraction of resorcinol, the N. F. method may be criticized with respect to the bromination procedure which directs that 20 cc. of water be added to the iodine flask after the liberation of bromine but *before* the addition of potassium iodide. By this procedure any bromine vapor above the liquid in the flask will be lost to whatever extent it occurs in the 20 cc. of air displaced by the water added at this point.

TABLE I.—RESULTS OF ANALYSES OF RESORCINOL PREPARATIONS

Preparation	Resorcinol Found, %	Recovery, %
Strong Resorcinol Paste, N. F. VIII	19.7	98.5
	20.0	100.0
	19.8	99.0
	19.8	99.0
	20.0	100.0
Ointment†	4.95	99.0
	4.89	97.8

† Formula: resorcinol 5%; cornstarch, 45%; zinc oxide and petroleum jelly, each 30%.

* Received July 26, 1950, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Federal Security Agency, Washington, D.C.

Satisfactory recoveries of resorcinol from both the previously mentioned ointment and strong resorcinol paste N. F., and elimination of mechanical difficulties were secured by following the simple procedure described below.

EXPERIMENTAL

A sample of paste or ointment, accurately weighed and containing 100–110 mg. of resorcinol, was introduced into a separatory funnel. Petroleum ether (10–15 cc.) was added and the funnel was swirled until the petrolatum was extracted from the sample and the solid ingredients had separated as a powder. Thirty cubic centimeters of 5% hydrochloric acid, accurately measured with a pipette, were introduced into the funnel which was then stoppered and shaken a few minutes. On standing, the phases rapidly separated. The aqueous layer, containing resorcinol, zinc chloride, and a small amount of suspended starch, was drawn off and filtered through a dry filter paper. A 20-cc. aliquot of the filtrate was pipetted into a 500-cc. iodine flask, a drop of methyl red, T. S. was added, and the solution was neutralized by the careful addition of 20% sodium hydroxide. Fifty cubic centimeters of 0.1 *N* bromine was then added from a pipette, and the determination was completed as described in the U. S. P. XIII monograph (3) for resorcinol.

Analytical results afforded by this procedure are listed in Table I.

SUMMARY

A procedure is described for the determination of resorcinol in pastes and ointments. The method gives good analytical results and has certain advantages over the procedure described in the N. F. VIII monograph on strong resorcinol paste.

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Synthesis of *dl*-Amphetamine Sulfate Labeled with C^{14}*,†

By JAMES W. WILSON, III

BECAUSE of the importance of Benzedrine‡ and Dexedrine‡ in medical practice, it was considered worth while to attempt to gain further insight into the mode and site of action of drugs of this type. For this purpose, we have prepared 279 mg. of *dl*-amphetamine neutral sulfate labeled with C^{14} in the position adjacent to the benzene ring.

The method of synthesis is outlined in Eq. 1. The methylene-labeled phenylacetic acid (1) was obtained from Tracerlab, Inc. It had a specific activity of approximately 1 μc./mM and after combustion and conversion to barium carbonate, showed 9.2 × 10^5 cts./sec./mg. of phenylacetic acid under standard conditions, using an end window Geiger-Müller counter.

(1)

The synthesis was carried out without isolating the intermediate products. The method employed in the synthesis of the phenylacetone was essentially that of Walker and Hauser (2). The ketone was converted to the oxime in an aqueous medium using an excess of hydroxylamine hydro-

* Received June 6, 1950, from Smith, Kline & French Laboratories, Philadelphia, Pa.
† The synthesis was carried out using the facilities of Tracerlab, Inc., of Boston, Mass. The cooperation and assistance of Dr. Melvin Fields of Tracerlab and Dr. Glenn E. Ullyot of Smith, Kline & French Laboratories are gratefully acknowledged.
‡ Smith, Kline & French Laboratories' trade mark names for racemic amphetamine sulfate, and dextro amphetamine sulfate, respectively.

chloride and sodium hydroxide (3). Reduction of the oxime was carried out in alcohol at atmospheric pressure and room temperature using a Raney nickel catalyst.

The over-all yield of neutral sulfate from the acid was 38 per cent. A sample was combusted, converted to barium carbonate, and counted under the same conditions used in counting the acid. It showed a specific activity of 7.55×10^3 cts./sec./mg. of the neutral sulfate. A small sample of the product was also converted to the benzoyl derivative. This compound gave no mixture melting point depression with an authentic sample of the derivative (4).

EXPERIMENTAL

A solution of 544 mg. of the methylene-labeled phenylacetic acid in 2.0 ml. of purified thionyl chloride was heated at gentle reflux for two hours. The thionyl chloride was removed by distillation *in vacuo*, after which dry benzene and dry toluene were successively added and removed *in vacuo*. The yellow acid chloride was heated at reflux for one and one-half hours with 10 ml. of an ether solution of the magnesioethoxy derivative of diethyl malonate, prepared according to the procedure of Walker and Hauser (2). The solution was cooled to 5° and hydrolyzed by the slow addition of 10 ml. of 10% sulfuric acid. The resulting diethyl phenylacetomalonate was taken into 40 ml. of ether by continuous extraction and the ether was removed by distillation. Hydrolysis and decarboxylation of the crude ketoester was effected by heating for five hours at gentle reflux with 4.5 ml. of glacial acetic acid, 1.5 ml. of water and 0.5 ml. of sulfuric acid. After the addition of 12 ml. of 20% sodium hydroxide and 20 ml. of water, the ketone was taken into 40 ml. of ether using a small continuous extraction apparatus. The ether was removed and the residual ketone was stirred vigorously at 40° with a solution of 300 mg. of hydroxylamine hydrochloride in 3 ml. of water, while 2.2 ml. of 4.02 *N* sodium hydroxide solution was added in a period of twenty minutes. After stirring at 40° for an additional thirty minutes, the addition of hydroxylamine hydrochloride and sodium hydroxide was repeated. The solution was neutralized with 0.6 ml. of glacial acetic acid and the oxime extracted into 30 ml. of ether. The ether was again removed by distillation and the oxime was dissolved in 7 ml. of alcohol. This solution was hydrogenated at room temperature and atmospheric pressure using a Raney nickel catalyst and a magnetic stirrer. The hydrogenation was complete in four hours and the uptake of hydrogen corresponded to 1.86 mM of oxime. The mixture was centrifuged and the alcohol solution removed. The catalyst was washed with three 3-ml. portions of ether and this wash solution was added to the alcohol solution. After neutralizing with 0.43 ml. of 3.42 *N* sulfuric acid, the mixture was cooled to −15° and the precipitated *dl*-amphetamine sulfate was separated by centrifuging. The precipitate was washed with three 5-ml. portions of ether and dried to constant weight *in vacuo*; the yield was 279 mg. (38% over-all). The N-benzoyl derivative melted at 132–134° and did not depress the melting point of an authentic sample (4).

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

1,1,1 Tribromoethane
Tri-(*o*-isocyanophenyl)methane
3-Methylcoumarone
ω-Hydroxydecanoic acid
3-Hexene-1-ol
5-Methylhexane-1-al
1-Methyl-4-methylol-glyoxaline
1-Methyl-4 methylol-imidazole
Hexokinase, crystalline
Myosin
Sabinene
β-Dimethylcholine
Primula acid
Cyanuric cyanide
Jaluronic acid
Nucleohistone
Psothanol
Methyl taumate
ω-Hydroxypropadrine

The Study of the Absorption of Sodium Ions in the White Rat Using Radioactive Tracer Techniques* †

By WILLIAM L. MADISON‡ and JOHN E. CHRISTIAN

The absorption of sodium ion in the rat has been studied after introduction into the gastrointestinal tract and after subcutaneous injection using radioactive sodium-24 and 22 labeled NaCl. By these methods the effect of various osmotic pressures, various *p*H's, and of certain astringent materials on normal absorption from these areas was determined.

PREVIOUS to the advent of radioactive tracers, studies of many fundamental biological processes were limited by the use of involved procedures with which it was not often possible to obtain high degrees of accuracy. Investigations involving studies of absorption were no exception. Today, however, advances in the field of radioactive isotopes production have made many isotopes so readily available that they are finding innumerable applications in virtually every field of scientific research. The fact that tracer atoms such as sodium-22 and sodium-24 are chemically identical with sodium-23 atoms makes them physiologically compatible to the extent that they may be used to excellent advantage as tracers in studies of biological phenomena, like absorption.

A survey of the literature (1–13) revealed that, while some scientific effort has been directed toward studying absorption processes in the animal body, the information reported is inconclusive and incomplete. Little attention has been given to the effects of physical factors on absorption, and even less attention has been directed toward studying the effects of specific substances on patterns of absorption.

This study was undertaken, therefore, to investigate the absorption of sodium ions, and the effect of certain factors on such absorption from a subcutaneous area, and from the gastrointestinal tract of the white rat.

EXPERIMENTAL

For the work involving absorption from a subcutaneous area, isotopes of sodium in the form of $Na^{22}Cl$ or $Na^{24}Cl$ were used as tracers. Both isotopes were standardized in the same manner. The specific activity of the radioactive salt was determined, and for use in experimentation, a sample calculated to have an activity of approximately 100,000 counts per minute[1] was taken from the original solution. This sample was transferred to a porcelain crucible, and an amount of pure, unlabeled NaCl was added to bring the combined weight of salt equal to exactly 30 mg. This addition of plain salt was performed to insure the same amount of NaCl being present in all samples. The resulting salt solution was then dried under an infrared lamp at 90°, and subsequently cooled to room temperature. To the salt contained in the crucible, exactly 0.5 ml. of boiled, cooled, distilled water was added, and the solution of the salt accomplished by gentle agitation.

For purposes of carefully standardizing the sample, 0.1 ml. of the above solution was transferred to a 25.0-ml. volumetric flask and diluted to mark with distilled water. Five-tenths milliliter of the resulting solution was transferred to a standard-sized filterstrip which was dried, scaled, and counted in an identical manner as that described later for the blood samples.

The white albino rat was selected for this work. No discrimination was made between sexes, and the weight of animals used was 200 ± 5.0 Gm. The animals were anesthetized by intraperitoneal injection of an aqueous solution of Amytal sodium containing 5.0 mg. for each 100 Gm. of body weight. The animal was placed on its back in a shallow Pyrex dish and its legs taped to the bottom of the dish with adhesive tape.

The remainder of the radioactive solution was drawn up into a syringe, and the injection accomplished by pulling up the loose skin of the rat at the ventral junction of the hind leg and lower abdomen and injecting subcutaneously.

At the end of five, fifteen, twenty-five, thirty-five, and forty-five minutes after injection, 0.7 ml. of blood was taken from the heart by heart puncture using a syringe rinsed with a 10% solution of sodium citrate and this blood transferred to a "well" in a porcelain spot-test plate.

Five-tenths milliliter of each radioactive blood sample was transferred to a standard-sized filterstrip 2 in. wide by 2 1/2 in. long and dried under an

* Received May 5, 1950, from the Purdue University School of Pharmacy, Lafayette, Ind.

Presented to the Scientific Section, A. Ph. A., Atlantic City meeting, May, 1950.

† Acknowledgment is made to Dr. D. J. Tendam of the Purdue Physics Department, and to Dr. P. H. Abelson of Carnegie Institution of Washington for their cooperation in supplying the radioactive tracers used in this work.

‡ Grateful acknowledgment is made to the American Foundation for Pharmaceutical Education, whose generous grant made this work possible.

[1] Equivalent to 100,000 counts per minute (c. p. m.) as determined with an Eck and Krebs type Geiger-Muller counter tube, with the radioactive sample absorbed on filter paper and wrapped around the counter tube.

infrared lamp at 90°. The filterstrips were sealed in between a piece of mimeograph paper and cellophane with Scotch Tape, and the activity determined under identical geometrical conditions and at the same time as the standardization samples.

By following the method outlined above, the normal absorption of the sodium ion from a subcutaneous area in the white rat was carefully determined. Following this, the effects of various osmotic pressures, various *p*H equivalents, and certain aluminum salts on the normal absorption pattern were determined. The results of this work are summarized in Table I and in Figs. 1, 2, 3, and 4.

The experimental procedure followed in studying the absorption of the sodium ion from the gastrointestinal tract of the white rat was comparable to that used in studying absorption from a subcutaneous area. There were, however, some differences, and these are briefly discussed.

The white rats used in this study weighed approximately 200 Gm., were starved for twenty-four hours previous to use, and were not anesthetized. The samples which were administered were calculated to have an activity of approximately 200,000 c. p. m. Variability from 200,000 c. p. m. in each sample was determined by counting a suitable aliquot and all results corrected to a time when the injection sample was equal to exactly 200,000 c. p. m.

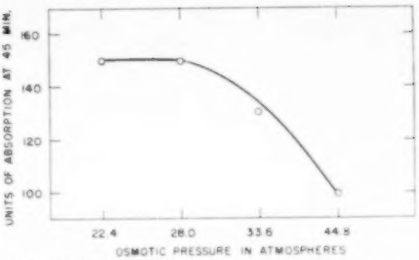

Fig. 1.—The effect of changes in osmotic pressure (of the sucrose solutions without the 30 mg. of added salt) on the normal absorption of the sodium ion from a subcutaneous area in the white rat.

(Normal absorption of the Na ion = 152.8 Units of Absorption.)

For purposes of administering the radioactive sample, the rat was held securely and a catheter introduced into the stomach. The 0.4 ml. of radioactive sample (prepared like those used in the sub-

TABLE I.—EXPERIMENTAL RESULTS INDICATING THE EFFECTS OF INCREASED OSMOTIC PRESSURE, CHANGES IN *p*H, AND CERTAIN ALUMINUM SALTS ON THE NORMAL ABSORPTION OF THE SODIUM ION FROM A SUBCUTANEOUS AREA IN THE WHITE RAT

Injection Sample	No. of Rats Used	Mean Units of Absorption[a] at 5 Min.	15 Min.	25 Min.	35 Min.	45 Min.
Na*Cl in distilled H_2O[b]	24	22.7	81.7	123.8	143.9	152.8
Na*Cl in sucrose[c] soln., 1.0 *M*	9	25.2	78.5	128.2	149.1	150.1
Na*Cl in sucrose soln., 1.25 *M*	9	22.9	77.8	123.9	148.2	152.8
Na*Cl in sucrose soln., 1.50 *M*	9	22.2	72.4	117.3	132.0	131.6
Na*Cl in sucrose soln., 2.0 *M*	10	19.8	65.5	103.0	107.1	109.2
Na*Cl in HCl, *p*H 1.0	12	13.7	57.5	107.7	126.5	129.0
Na*Cl in HCl, *p*H 2.0	8	19.6	67.5	118.3	133.6	137.0
Na*Cl in HCl, *p*H 2.5	9	22.1	78.2	123.9	147.0	150.8
Na*Cl in HCl, *p*H 3.0	10	22.5	78.7	122.4	140.5	146.8
Na*Cl in HCl, *p*H 4.0	10	22.8	76.8	123.6	143.7	147.9
Na*Cl in HCl, *p*H 5.0	9	21.7	78.8	123.9	148.2	152.9
Na*Cl in HCl, *p*H 6.0	8	22.3	79.2	121.7	143.4	150.1
Na*Cl in NaOH, *p*H 8.0	9	21.7	79.1	123.5	149.8	158.5
Na*Cl in NaOH, *p*H 9.0	9	23.3	78.7	124.8	151.1	149.6
Na*Cl in NaOH, *p*H 10.0	9	22.4	81.4	122.2	155.9	150.2
Na*Cl in NaOH, *p*H 11.0	9	34.5	78.4	133.0	161.6	160.1
Na*Cl in NaOH, *p*H 12.0	9	37.3	94.6	136.6	166.3	170.6
Na*Cl in Al. chloride[d] 0.10 *M* (1.35°$_c$)[e]	8	21.1	34.6	55.5	72.5	96.2
Na*Cl in Al. methionate 0.09 *M* (5.00°$_c$)	10	22.0	40.1	55.5	65.9	75.2
Na*Cl in Al. chloride 0.20 *M* (2.70°$_c$)	8	18.3	36.1	52.8	67.4	74.2
Na*Cl in Al. methionate 0.10 *M* (5.76°$_c$)	9	11.9	28.9	41.7	66.9	71.5
Na*Cl in Al. methionate 0.18 *M* (10.00°$_c$)	8	17.2	35.5	51.5	59.7	67.2
Na*Cl in Al. sulfate 0.10 *M* (3.42°$_c$)	8	15.8	31.3	40.6	51.7	66.4
Na*Cl in Al. methionate 0.20 *M* (11.50°$_c$)	9	12.9	28.0	42.9	61.9	65.9
Na*Cl in Al. chloride 0.37 *M* (5.00°$_c$)	10	12.5	32.5	45.4	55.9	62.1
Na*Cl in Al. sulfate 0.15 *M* (5.00°$_c$)	9	13.8	26.4	40.1	52.7	54.4
Na*Cl in Al. sulfate 0.20 *M* (6.85°$_c$)	8	11.9	25.9	42.3	41.7	41.1
Na*Cl in Al. chloride 0.74 *M* (10.00°$_c$)	9	12.5	29.7	36.5	37.7	40.2
Na*Cl in Al. sulfate 0.29 *M* (10.00°$_c$)	9	11.9	22.0	26.9	30.4	30.5

[*] The asterisk when used as a superscript to an atomic symbol denotes the radioactive isotope of the element (e.g., Na*).

[a] Arbitrary values used to express the absorption of the sodium ion, and obtained by applying the formula $(A/B) \times 500$, where A = actual c. p. m. recorded from the sample and corrected for background, and B = the standard in c. p. m. corrected for background.

[b] Normal absorption.—In order to determine the significance of the experimental results, all data were treated statistically.

[c] The osmotic pressures of the above four sucrose solutions, not including the 30 mg. of added salt used in the injection samples, were 22.4, 28.0, 33.6, and 44.8 atmospheres in the descending order in which they appear.

[d] The osmotic pressures and *p*H equivalents of each of the aluminum salt solutions were determined to discover whether they were of such a magnitude as to effect absorption; they were not. The osmotic pressure determinations were made using the Beckman apparatus (Steel, 1928). The *p*H equivalents were determined by the usual methods.

[e] Arranged in increasing order of effect on Na* absorption at the forty-five-minute time interval.

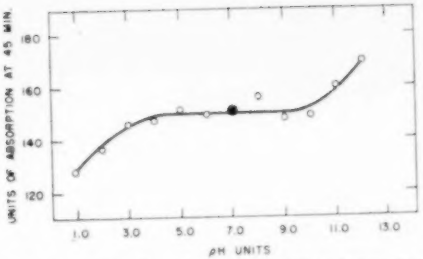

Fig. 2.—The effect of changes in *p*H on the normal absorption of the sodium ion from a subcutaneous area in the white rat.
(● = Normal absorption of the Na ion.)

cutaneous study) remaining after the removal of the 0.1 ml. used for standardization, was introduced by hypodermic syringe through the catheter into the animal's stomach, followed by 1.0 ml. of distilled water to insure thorough emptying of the catheter. The rat was placed in a holder designed to limit the

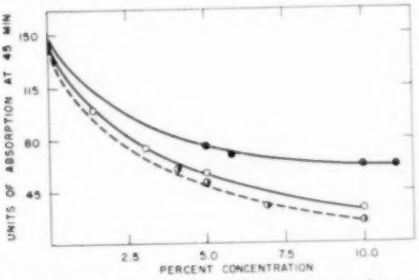

Fig. 3.—The effect of certain aluminum salts, expressed in per cent concentration, on the normal absorption of the sodium ion from a subcutaneous area in the white rat.
——● = Aluminum methionate.
——○ = Aluminum chloride.
- - - ○ = Aluminum sulfate.

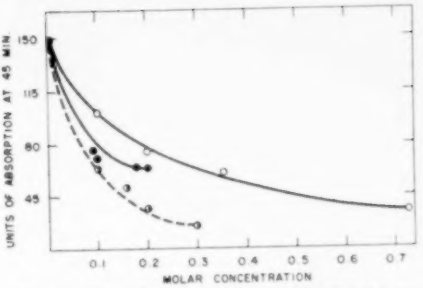

Fig. 4.—The effect of certain aluminum salts, expressed in molar concentration, on the normal absorption of the sodium ion from a subcutaneous area in the white rat.
——○ = Aluminum chloride.
——● = Aluminum methionate.
- - - ○ = Aluminum sulfate.

freedom of the animal and to present the full length of the tail. The rat in holder was placed behind a lead plate of 5 cm. thickness with a hole of 1.5 cm. in diameter at the height of the base of the tail. The tail of the rat was drawn through the hole in the lead plate and fastened longitudinally and directly below a Geiger-Müller tube (Eck and Krebs type).

Radiations emanating through the tail of the animal were counted for a period of two to two and one-half hours. The one-minute counts from the tail of the animal were averaged, corrected for background, and the units of absorption calculated. The results of this study are presented in Table II and in Fig. 5.

SUMMARY AND CONCLUSIONS

1. Two techniques have been successfully established for determining the absorption pattern for the sodium ion in the rat using radioactive tracers. The first method consists of injecting Na^{22} or Na^{24}-labeled sodium chloride sub-

TABLE II.—EXPERIMENTAL RESULTS INDICATING THE EFFECTS OF CERTAIN ALUMINUM SALTS ON THE NORMAL ABSORPTION OF THE SODIUM ION FROM THE GASTROINTESTINAL TRACT OF THE WHITE RAT

Min. after introduction	Dist.[b] H_2O	Mean Units of Absorption[a] for NaCl Dissolved in: 0.37 *M* (5.00%) $AlCl_3$	0.74 *M* (10.00%) $AlCl_3$	0.18 *M* (10.00%) Al. Methionate	0.29 *M* (10.00%) $Al_2(SO_4)_3$
20	487	550	212	100	125
30	825	737	387	212	265
40	1100	937	550	313	435
50	1310	1112	725	413	583
60	1450	1250	875	537	693
70	1588	1375	962	675	767
80	1662	1500	1039	813	900
90	1725	1564	1125	875	985
100	1740	1600	1185	975	1061
110	1772	1611	1225	1050	1155
120	1800	1635	1275	1100	1185
130	—	1675	1362	1139	1235
No. of rats used	17	8	10	8	8

[a] In order to determine the significance of the experimental results, all of these results were treated statistically. These mean units of absorption were calculated by means of the same formula used in the previous work.
[b] Normal absorption.

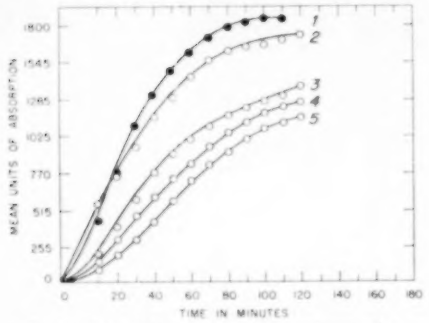

Fig. 5.—The effect of certain aluminum salts on the normal absorption of the sodium ion from the gastrointestinal tract of the white rat.

1, Na*Cl in 0.4 ml. dist. water (normal absorption).
2, Na*Cl in 0.4 ml. 5% $AlCl_3$.
3, Na*Cl in 0.4 ml. 10% $AlCl_3$.
4, Na*Cl in 0.4 ml. 10% $Al_2(SO_4)_3$.
5, Na*Cl in 0.4 ml. 10% aluminum methionate.

cutaneously at the ventral junction of the lower abdomen and hind leg, and "counting" blood samples from the animal. The second method consists of introducing the labeled salt into the gastrointestinal tract by stomach catheter, and counting ionizing radiations emanating from the tail of the animal. Both methods were found to be convenient and accurate.

2. The effects of various osmotic pressures on the normal absorption of the sodium ion in the white rat were determined by subcutaneously injecting solutions of sucrose along with the radioactive sodium. Sucrose solutions having osmotic pressures of 28.0 atmospheres or less were found to have little effect on the normal absorption, while those having osmotic pressures of 33.6 atmospheres or greater were found to decrease the absorption of the sodium ion from the normal.

3. The effects of various *p*H equivalents on the normal absorption of the sodium ion in the white rat were determined by subcutaneously injecting solutions of HCl or NaOH having various *p*H values. Little effect upon the normal absorption of the sodium ion was observed from *p*H 2.5 to 10.0, inclusive. However, solutions of the acid having a *p*H of 1.0 and 2.0 caused a decrease in the absorption of the sodium ion, while solutions of the base having a *p*H of 11.0 or 12.0 caused an increase in the absorption.

4. Aluminum chloride, sulfate, and methionate, in concentrations of 0.2 *M*, 0.1 *M*, 5.0 per cent, and 10.0 per cent, were injected subcutaneously with the labeled sodium chloride and their effect in relation to the normal absorption of the sodium ion studied. These aluminum salt solutions all caused a decrease in the normal absorption pattern as determined by the method used. Expressing the results in molar concentrations, aluminum chloride caused the least inhibition to absorption, while aluminum sulfate caused the greatest decrease in absorption, and aluminum methionate was intermediate between the two. Expressing the results in per cent concentrations, aluminum methionate showed the least power of inhibition, aluminum sulfate the greatest, and aluminum chloride was intermediate between the two.

5. Aluminum chloride in concentrations of 5.0 per cent and 10.0 per cent, and aluminum sulfate and methionate in 10.0 per cent concentrations were administered with labeled NaCl by means of a stomach catheter to the white rat, and their effect in relation to the normal absorption pattern for the sodium ion, as determined by this method, studied. These aluminum salt solutions all caused a depression of the normal absorption pattern. Aluminum chloride caused a greater depression in 10.0 per cent concentration than in 5.0 per cent concentration, but caused less depression than the other two aluminum salts of the same concentration. Between aluminum sulfate and aluminum methionate, the latter salt caused the greatest depression of the sodium ion absorption as determined by the method used.

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Tissue Distribution of S^{35} in the Rat Following Oral Administration of Labeled Sulfanilamide*

By E. G. FINGL,† J. E. CHRISTIAN, and L. D. EDWARDS‡

Sulfur-35 was found to be relatively uniformly distributed in the animal body at one, two, four, eight, and twenty-four hours after administration of labeled sulfanilamide. The same was true two hours after the last of 16–18 doses at four-hour intervals. The tissues concerned with excretion and detoxification tended to show, particularly at the later time intervals, concentrations slightly higher than the other tissues. There is no evidence of extensive binding of sulfanilamide by the tissues.

THE IMPORTANCE of the relationship between the characteristic tissue distribution of a medicinal agent and its eventual therapeutic effectiveness cannot be denied. It is unfortunate that a property of such importance defies attempts at thorough investigation. Quantitative, chemically intact recovery of the compound from the tissues is one of the most difficult hurdles in such studies, as is quantitative determination of the compound in the presence of impurities which may obscure the end point of the assay. With the recent increase in availability and decrease in cost of production of isotopes, both stable and radioactive, a new approach to tissue distribution studies has become practical.

The initial studies of Marshall, Emerson, and Cutting (1, 2) indicated that sulfanilamide was uniformly distributed throughout the entire body water of the animal, resembling urea and ethanol. However, accumulation of additional data, such as that of Waterhouse and Shannon (3), has shown that while sulfanilamide readily penetrates all tissues and is relatively uniformly distributed, the distribution is not dependent solely upon the distribution of water. Davis (4, 5) has demonstrated the binding of sulfanilamide to the albumen fraction of the plasma protein, and Riser and Valdiguie (6) have noted fixation of this compound by muscle and brain. Hansen (7) has reported higher concentrations of sulfanilamide in the red blood cells than in the plasma, but believed that the explanation depends upon differences in diffusion and absorption rather than in a loose chemical or physical bond between the tissue and sulfanilamide. These observations, as well as others, such as that of Cornbleet (8) in which a sweat bath resulted in the reappearance of sulfanilamide in the blood several days after its initial disappearance, suggest that some unusual features of sulfanilamide distribution are yet to be uncovered.

EXPERIMENTAL

Synthesis of Labeled Sulfanilamide[1]

Labeled sulfanilamide was synthesized by the method developed by Christian and Bachmann (9).[2] Ten milliliters of $H_2S^{35}O_4$ in 0.25 *N* HCl (assayed as 20 μc./19.3 ml. seven days previously at Oak Ridge[3]) was transferred by pipette to a 30-ml. beaker and concentrated on a steam bath. When the volume had been reduced to approximately 0.5 ml., the residue was chilled and 1.6 Gm. of concentrated H_2SO_4 added. Four grams of acetic anhydride was then added gradually with further chilling. To this mixture 2.0 Gm. of acetanilid was added and the mixture heated on a steam bath for thirty minutes. N-acetylsulfanilic acid separated. The reaction mixture was cooled, diluted with 10 ml. of acetone, and filtered with suction. The residue was washed with chilled acetone and then with ether, and the ether allowed to evaporate. When the residue had dried, it was neutralized with an equivalent of NaOH dissolved in 2 ml. of water and sufficient acetone added to completely precipitate the salt. The residue was collected by suction and washed with ether. After the ether had evaporated, the salt was dried over concentrated H_2SO_4. Yield: 3.4 Gm. (97% theoretical).

The sodium salt of N-acetylsulfanilic acid was divided into two portions for the succeeding steps of the synthesis. The crude yields were then combined for the purification.

Sodium salt (1.7 Gm.) of N-acetylsulfanilic acid was rubbed with 3.4 Gm. of PCl_5 in a small beaker. Gentle heat was applied to insure complete reaction, a homogeneous liquid mass indicating completion. The mixture was allowed to cool and several milliliters of cracked ice and water added to decompose the excess PCl_5. The water was decanted and 3.4 ml. of 28% NH_4OH added to the sulfonyl chloride and the mixture warmed on a steam bath to bring about complete reaction. When the mixture had cooled, the residue was collected by suction and washed with several 2-ml. quantities of cold water. The acetylsulfanilamide was then hydrolyzed by

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‡ We are indebted to Dr. D. J. Tendam of the Physics Department of Purdue University for maintenance of the measuring instruments used in this research.

[1] An additional quantity of labeled sulfanilamide was synthesized by Dr. R. O. Bachmann of the Department of Pharmaceutical Chemistry, School of Pharmacy, Purdue University.
[2] Reported by essentially the same method after this work was completed [Klotz, I. M., and Melchior, J. B., *Arch. Biochem.*, **21**, 35(1949)].
[3] The isotopes used in this research were obtained on allocation by the U. S. Atomic Energy Commission.

warming with 3.4 ml. of 10% HCl on a steam bath until solution occurred. The solution was then cooled and carefully neutralized with 10% NaOH until just alkaline to litmus. Upon cooling, the sulfanilamide that crystallized was collected by suction. This crude yield was combined with the similar product from the other half of the synthesis. Recrystallization from water was repeated until a product melting at 164.5° [mmp(K)] was obtained. The filtrates from the crude yields and from all subsequent recrystallizations were combined and 2.0 Gm. of U. S. P. Reference Sulfanilamide added. Recrystallization from water was repeated until a second pure, but less active, fraction of labeled sulfanilamide was obtained. Yield: 25–35% theoretical.

Activity of the first fraction: theoretical—3.7 μc./mg.; observed—1,300,000 c./m./mg. (Determined as $BaSO_4$, corrected for self-absorption to zero absorber, at 12 mm. from a Victoreen bell-shaped Geiger-Müller tube having a mica window thickness of 2.8 mg./cm.[3])

Assay of Tissue for Sulfur-35

Each sample for assay was placed in a 100-ml. Pyrex beaker. Liquid samples were measured by pipette; tissue samples were weighed on a Torsion balance to 0.01 Gm. Four milliliters of 10% NaOH (10) and sufficient distilled water to cover the sample were added and gentle heat applied until the sample was in a homogeneous, liquid state. Ten milliliters of Benedict-Denis reagent (11) was added and the mixture heated to dryness. The sample was then placed in a hot air oven at 180° overnight to insure complete dryness. The following day the sample was heated on a hot plate at "low" and "medium" for ten minutes each, and then muffled in a muffle furnace at 500–550° for ten minutes. After removal from the furnace, the sample was again heated on hot plates at "medium" and "low" for ten minutes each. Immediately after removing the beaker from the second hot plate, 2.5 ml. of concentrated HCl was added and the sample allowed to cool. Sufficient Na_2SO_4 solution was added as carrier to yield a barium sulfate sample weighing 25–35 mg. Distilled water was added to bring the volume to approximately 45 ml. and, if necessary, gentle heat applied to complete solution. Three milliliters of saturated solution of picric acid (12) was added, the solution heated to boiling, and 2 ml. of 5% $BaCl_2$ added all at once. The beaker was covered with a watch glass and the solution boiled gently for five to ten minutes. The barium sulfate precipitate was collected four to twelve hours later on a small filter paper disk, washed with acetone, dried under an infrared lamp, and weighed immediately to 0.1 mg.

The activity of each sample was determined by means of a single count of approximately ten minutes, corrected for background and self absorption and compared with a suitable standard. Self-absorption correction was determined by applying factors derived from data supplied by Libby (13). The standard consisted of duplicate aliquots of the administered solution prepared in the same manner as the tissue samples and having an observed activity of 500–1,000 c./m.

Bone samples were assayed in a similar manner, with the exception that the preliminary digestion was accomplished with dilute HCl. After addition of the Benedict-Denis reagent, NaOH solution was added until the *p*H of the sample approximated that of the tissue samples.

The above procedure was tested for quantitative recovery of S^{35} from labeled sulfanilamide both with and without the presence of animal tissue. The mean error of eight determinations without animal tissue was 2.2 ± S. E. O. 37%, while that for 16 determinations with animal tissue was 2.2 ± S. E. O. 36%. Tests for quantitative recovery of the total dose administered to an animal and for accuracy of the self-absorption correction also indicated satisfactory accuracy.

Tissue Distribution of S^{35} in the Rat Following Oral Administration of Labeled Sulfanilamide

Procedure.—Healthy, young male albino rats (Wistar-Purdue strain), weighing 125–150 Gm., were used throughout this work. Food was withheld from each animal for a period of eighteen hours before use; water was permitted. Each animal received 100 mg./Kg. of labeled sulfanilamide orally, as a 0.75% solution in distilled water. The solution was administered by means of a protected 19-gauge 3-in. hypodermic needle inserted down the esophagus and delivered from a previously calibrated syringe.

The animal was then placed upon a wire-mesh shelf suspended near the bottom of a half-gallon glass funnel until time for sacrifice. Water was again permitted, but food was withheld for an additional eight hours. Urine and feces were collected without attempt at separation. Twenty minutes before the time for sacrifice 50 mg./Kg. of pentobarbital sodium was administered intraperitoneally as a solution in distilled water containing 10 mg./ml. When anesthetized, the animal was secured, ventral surface up, on another shelf suspended near the top of the funnel. The container for collection of the excreta was replaced by another container for the collection of the perfusate, and the animal sacrificed by the viviperfusion technique of Copp and Greenberg (14). The only significant modification of the technique was reduction of the perfusion time to two minutes. Tissue samples were dissected, freed from as much extraneous tissue as possible, and transferred to ground glass-stoppered weighing bottles. Duplicate aliquots of an alkaline digest of tissues pooled from ten animals were assayed for S^{35} by the method outlined.

Results.—Determinations were made at one, two, four, eight, and twenty-four hours after the administration of a single dose and two hours after the last of 16–18 doses at four-hour intervals. The results are tabulated in Table I. The plasma concentrations are expressed as: mg.% S^{35}, while the tissue concentrations are expressed as ratios: mg.% S^{35} tissue/mg.% S^{35} plasma. The values reported after single dose administration are based upon the assay of duplicate aliquots of tissues pooled from ten animals, while the values reported after prolonged administration are based upon the assay of tissues from two animals assayed individually. Tissues from two animals were also assayed individually after single doses, but for the sake of clarity these values have been omitted from the table; they did not differ significantly from those reported.

TABLE I.—TISSUE DISTRIBUTION OF S^{35} IN THE RAT AT INTERVALS AFTER THE ORAL ADMINISTRATION OF A SINGLE DOSE AND AFTER PROLONGED ADMINISTRATION OF LABELED SULFANILAMIDE

Tissue	Hours After Administration of Single Dose					Two Hours After Prolonged Administration
	1	2	4	8	24	
	Mg. % S^{35}					
Plasma	1.18	1.12	1.20	0.36	0.02	1.46
	Mg.% S^{35} tissue/mg.% S^{35} plasma					
Stomach	1.71	1.51	0.83	1.11	1.96	0.74
Small intestine	0.52	0.78	0.51	0.64	0.77	0.53
Large intestine	0.73	0.99	0.57	0.94	2.78	0.65
Spleen	0.90	1.15	0.74	1.14	2.27	0.95
Pancreas and fat	0.57	0.64	0.51	0.72	0.72	0.70
Kidney	1.04	1.09	0.87	1.88	2.78	1.06
Adrenal	...	0.51	0.37	0.47	...	...
Liver	0.78	1.13	0.71	1.05	1.08[a]	0.84
Testis	0.80	1.10	0.80	1.14	0.85[a]	0.90
Heart	0.50	0.72	0.41	0.66	0.66[a]	0.59
Lung	0.58	0.81	0.41	0.83	1.13	0.63
Gastrocnemius	1.01	1.04	0.78	1.11	0.92[a]	0.94
Femur	0.66	0.66	0.58	0.72	0.68[a]	0.63
Skin and hair	1.08	0.87	0.73	0.94	0.94[a]	1.05
Brain	0.62	0.87	0.56	0.58	0.56[a]	0.70
Blood	1.26	1.31	...	1.58	...	1.40

[a] Indicate values based upon individual assays.

DISCUSSION

Sulfur-35 is found to be relatively uniformly distributed throughout the entire animal body at each of the time intervals studied. The tissues concerned in excretion and detoxification tend to show slightly higher concentrations than the others, particularly at the later time intervals. The slightly lower concentrations found in the heart and lungs probably reflect the effect of the perfusion procedure.

Williams (15) has demonstrated the metabolism of sulfanilamide in the rabbit to 3-hydroxy-sulfanilamide as well as acetylsulfanilamide, and therefore, the measurement of S^{35} is probably a measure of these three compounds simultaneously. However, since Marshall, Cutting, and Cover (16) have reported that only a small amount of sulfanilamide is acetylated by the rat in the first six hours, the distribution of S^{35} at the early time intervals may be assumed to be essentially that of sulfanilamide, and the alterations in the distribution at the later time intervals reflect the presence of the metabolic products and/or binding of the compounds to the tissue. The distribution after prolonged administration tends to support the conclusion that sulfanilamide is not extensively accumulated.

SUMMARY AND CONCLUSIONS

1. The tissue distribution of S^{35} in the rat following oral administration of labeled sulfanilamide has been studied at one, two, four, eight, and twenty-four hours after administration of single doses, and two hours after the last of 16 to 18 doses at four-hour intervals.

2. S^{35} was found to be relatively uniformly distributed in the animal body throughout the series of determinations. The tissues concerned in excretion and detoxification tended to show, particularly at the later time intervals, concentrations slightly higher than the other tissues.

3. Interpretation of the S^{35} distribution in terms of sulfanilamide distribution indicates relatively uniform distribution throughout the entire animal body. There is no evidence of extensive accumulation of sulfanilamide by the tissues.

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Factors Affecting the Rutin Contents of Dried Buckwheat Meals*

By J. NAGHSKI, C. F. KREWSON, W. L. PORTER, and J. F. COUCH†

The distribution of rutin in Tartary and Japanese buckwheat was determined at three ages of the plant. Fractional drying and screening produce a leaf meal of high rutin content. Wilting in the sun was evaluated as an adjunct to artificial drying of buckwheat for production of dried meals.

INVESTIGATIONS of domestic plants containing rutin revealed that buckwheat contains enough to be a commercial source of the glycoside. Preliminary studies (2) showed that the major portion of the rutin was in the leaves and blossoms of the immature plant and that the stems contained only a small fraction. These results suggested that rutin could be concentrated by the preparation of a leaf meal. It was possible to separate the buckwheat plant into leaf and stem fractions by applying the principles used in the preparation of leaf meals from vegetable wastes (1). In that procedure, the plant is dried until the leaves are brittle, and then the leaves are separated from the partly dried stems by screening. It was found that rutin is easily destroyed and that the drying process has to be carried out under strictly controlled conditions. Eskew and co-workers investigated this problem and worked out in detail the procedure for producing a dried leaf meal (4, 5, 7). They found that even under the best conditions of fractional drying, 25 to 35 per cent of the rutin was lost. When the buckwheat was totally dried, the destruction was somewhat greater.

In a study of the influence of various agronomic factors on the rutin content of buckwheat, it was discovered that the Tartary variety (*Fagopyrum tataricum*) was richer in rutin than the Japanese (*F. esculentum*) (3). Since the former variety appeared to be richer in leaf material, a study was undertaken to determine the distribution of rutin in the buckwheat plant and the factors that influenced this relationship. Another factor studied was the effect of wilting or sun-drying on the rutin content of dried buckwheat.

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DISCUSSION OF EXPERIMENTS

Distribution of Rutin in the Buckwheat Plant.—Tartary and Japanese buckwheat were grown on well-fertilized soil with the plants spaced at 2-in. intervals in rows 12 in. apart. The harvested green plants were separated into two fractions: (*a*) stems, and (*b*) leaves and blossoms. Petioles were considered as part of the leaves, since they are not separated from the leaves by the mechanical separation (4). The samples were analyzed for crude or corrected rutin by the method of Naghski, *et al.* (6). Table I shows the results obtained at three ages. About 81–89% of the rutin is localized in the leaves and blossoms, which constitute from 45 to 70% of the dry weight of the plant, depending on its age. The leaf fraction from the Tartary buckwheat is richer in rutin than that from the Japanese. The data in Table I show that, at the proper age of the plant, the rutin can be concentrated as much as 78% by discarding the stems This value, of course, is theoretical, and would not be attained in a mechanical process. During the course of this study, numerous buckwheat leaf meals and corresponding fresh plants were analyzed. Since only limited data are available in the literature on how effectively rutin is concentrated by producing leaf meals, some results obtained with selected samples are presented in Table II.

Since corresponding whole meals were not available for analysis, the rutin content was approximated by calculation on the basis of discarding 37% of the dry weight as stems containing 0.3% rutin. Although the quantity of stems removed in individual runs will vary from 37%, the expected average for commercial production will be near this value, which Phillips, *et al.* (7), found to be the average value from a number of typical runs. The value of 0.3% for the rutin content was the average of a number of dried and partially dried stems (6). Individual samples ranged from 0.0 to 0.57% rutin.

On this basis, the leaf meals were about 50% richer in rutin than the corresponding meals made from the whole plant. This richer meal increased the capacity of the equipment for extracting rutin. Furthermore, in fractionally dried plants, this concentration was more than sufficient to offset the loss of rutin incurred in the drying process, since the leaf meal was richer in rutin than the starting fresh plant. When the plant was totally dried, the loss of rutin caused by drying was higher, and the leaf meal produced did not have as high a rutin content as the fresh plant. Leaf meals containing 4 to 5% rutin can be produced easily, and leaf meals containing as much as 6.7% rutin have been produced at this laboratory. Meals of this quality would probably compete favorably with some of the newer sources of rutin, now imported in limited commercial quantities.

TABLE I.—DISTRIBUTION OF RUTIN IN TARTARY AND JAPANESE BUCKWHEAT PLANTS AT DIFFERENT AGES[a]
(Average Values on Moisture-Free Basis)

Age, Days → / Species →	31		38		45	
	Tartary	Japanese	Tartary	Japanese	Tartary	Japanese
Whole Plant						
Moisture, %	92.0	91.4	92.1	88.5	86.7	83.0
Dry weight of plant, Gm.	1.0	1.6	1.9	3.2	4.8	7.7
Crude rutin, %	3.8	3.3	4.3	2.7	3.6	1.9
Crude rutin, mg./plant	38.0	52.8	81.7	86.4	172.8	146.3
Leaves and blossoms, % of whole plant	70	60	56	51	45	53
Leaves and Blossoms						
Moisture, %	88.1	87.5	89.3	85.5	85.6	81.6
Crude rutin, %	4.7	4.5	6.3	4.7	6.4	3.2
Crude rutin, mg./plant	32.9	43.7	66.2	76.6	139.5	129.3
Crude rutin, % of total in plant	87	83	81	89	81	88
Increase in concentration of rutin over that in whole plant, %	24	36	47	74	78	68

[a] Plants grown near Philadelphia in 1946. Fertilized at rate of 1,000 lb. of 5–10–5 per acre.

Similar results were obtained with the Japanese variety. Since it is not as rich in rutin as the Tartary variety, however, and it is not being used commercially, the data are not presented.

Effect of Wilting or Sun-drying on Rutin Content of Buckwheat.—Since artificial drying is expensive, the effect of sun-drying was investigated. Unlike most plants, buckwheat does not sun-cure readily. Although the leaves dry quickly, the stems retain their moisture for a long time. Samples[1] of whole Tartary plants were exposed to sunshine for three days. At the end of each day, the plants were separated into a leaf fraction and a stem fraction, and oven-dried. Table III shows the moisture contents.

TABLE II.—RUTIN CONTENT OF LEAF MEAL PRODUCED BY FRACTIONAL AND TOTAL DRYING OF TARTARY BUCKWHEAT PLANTS[a]
(Moisture-Free Basis)

Sample No.	Corrected Values for Rutin in—Fresh Plant (A), %	Leaf Meal (B), %	Whole Meal[b] (C), %	Increased Concn. of Rutin in Leaf Meal,[c] %
Fractionally Dried				
1	4.64	4.89	3.19	53
2	5.22	5.38	3.50	54
3	3.97	3.26	2.16	51
4	4.49	4.74	3.10	53
5	3.49	3.53	2.33	52
Totally Dried				
6	3.89	3.46	2.29	51
7	4.81	4.03	2.65	52
8	2.89	1.73	1.20	44
9	3.28	3.15	2.09	51

[a] Samples obtained during the investigations on buckwheat drying by Phillips, *et al.* (7).
[b] Calculated on the basis of discarding 37% of the dry weight as stems containing 0.3% rutin.
[c] $\frac{B - C}{C} \times 100$.

TABLE III.—EFFECT OF SUN-DRYING ON MOISTURE CONTENTS OF TARTARY BUCKWHEAT PLANTS[a]

Time Exposed to Sun, Hr.	Moisture Content of—Leaves, %	Stems, %	Whole Plant, %
0	87.5	93.3	91.3
7.5	24.4	88.2	79.5
15.5	13.0	85.5	75.2
20	12.1	85.0	73.3

[a] Experiment conducted September 12–14, 1947.

The results show how misleading the over-all moisture can be in regard to the dryness of the plant. The most rapid drying took place during the first day, and the greatest loss of moisture was in the leaves. The ratio of leaf to stem (dry weight) in these plants was 1:1, indicating the magnitude of loss of moisture from the leaves. The significance of this in the drying of buckwheat, especially in the preparation of leaf meal, is self-evident.

Another experiment was conducted to compare the rate of drying of young and old buckwheat plants. The old plants were badly lodged, so that

[1] In each wilting experiment described in this paper, replicate 200.0-Gm. samples were used.

TABLE IV.—EFFECT OF SUN-DRYING ON MOISTURE CONTENTS OF LEAVES AND STEMS OF OLD AND YOUNG TARTARY BUCKWHEAT PLANTS[a]

Time Exposed Sun, Hr.	Time Exposed Dark, Hr.	Moisture Content of—Young Plants—Leaves, %	Stems, %	Whole Plant, %	Old Plants—Leaves, %	Stems, %	Whole Plant, %
0	0	79.6	93.6	89.6	78.5	77.6	77.9
0	24[b]	36.8	90.3	86.5	45.7	65.0	61.7
6	0	37.0	90.1	83.8	29.7	66.0	55.6
12	16[c]	59.5	87.9	82.6	14.3	66.0	51.0

[a] Experiment conducted September 16–17, 1947.
[b] Dried at room temperature.
[c] Heavy dew during night.

only the top (18–24 in.) was suitable for use. Samples were exposed to the sun, then separated into leaf and stem fractions. The seeds in the old sample gave a little trouble after sun-drying, since they tended to drop off into the leaf fraction. The results are presented in Table IV.

The weather was not as hot as it was the previous week, and this is reflected in the poorer drying of the plants. A comparison can be made, however, between the rates of drying at the two ages of the plants. The leaves on the older plant, being smaller and thinner, dried much more rapidly. The stems from both ages dried at approximately the same rate.

Two experiments (Table V) showed that there was no appreciable loss of rutin during the exposure to sun for one or two days. However, plants that accidently were exposed to rain for thirty-eight hours before they were completely dry, lost almost 40% of their rutin when they were again exposed to sunshine. In this case, rain restored the leaves to normal turgor, and they evidently respired actively when subsequently exposed to sunlight, making the rutin susceptible to destruction.

TABLE V.—EFFECT OF SUN-DRYING ON MOISTURE AND RUTIN CONTENTS OF JAPANESE BUCKWHEAT PLANTS

Time Exposed			Crude Rutin[a]		
Sun, Hr.	Dark, Hr.	Moisture, %	A, %	B, %	Av. %
		July 10–12, 1946			
0	0	86.8	3.28	2.80	3.04
0	5	86.7	3.60	3.29	3.44
1	0	86.3	3.67	3.36	3.52
3	0	83.5	3.41	3.37	3.39
5	0	82.8	3.04	2.83	2.94
5	17	83.9	3.36	3.46	3.41
12	17	77.3	2.72	3.09	2.91
12	35	77.5	3.01	3.00	3.01
		August 6–8, 1946			
0	0	91.6	2.84	3.00	2.92
1	0	89.4	3.13	2.86	3.00
3	0	89.4	3.21	2.60	2.91
5	0	88.2	3.15	2.88	3.02
5	21[b]	90.3	3.06	3.67	3.37
13	38[b]	84.2	1.96	2.15	2.06

[a] Moisture-free basis.
[b] Continuous rain.

In another experiment, the plants were spread out in the dark for nineteen hours before exposure to sunlight. The results presented in Table VI show that after this treatment rutin was destroyed rapidly. Evidently the plant must be dried rapidly (especially the leaves) if rutin is to be preserved.

Investigation of large-scale wilting under field conditions was made by Phillips, *et al.* (7). They found that, under optimum conditions, wilting removes from one-third to one-half the moisture without any appreciable loss of rutin. If as much as one-half the moisture was removed, however, excessive loss of rutin occurred on drying. There were indications that wilting done too slowly destroyed considerable rutin. Although it is difficult to obtain uniform exposure to sunlight under field conditions, as pointed out by Phillips, *et al.*, their results agree closely with our laboratory-scale experiments cited above.

TABLE VI.—EFFECT OF STORING TARTARY BUCKWHEAT PLANTS IN THE DARK BEFORE SUN-DRYING ON RUTIN CONTENT

Time Exposed to Sun,[a] Hr.	Crude Rutin[b] A, %	B, %	Av., %	Apparent Loss, %
0	3.75	4.26	4.01	...
7.5	2.48	2.52	2.50	37.5
15.5	1.61	1.45	1.53	62.0

[a] Plants stored for nineteen hours in dark at room temperature before exposure to sunlight (September 12–14, 1947).
[b] Moisture-free basis.

SUMMARY

About 80 to 90 per cent of the rutin of the buckwheat plant is localized in the leaves and blossoms, which constitute from 45 to 75 per cent of the dry weight of the plant. Fractional drying produces a leaf meal with 50 per cent higher rutin content than corresponding whole plant meals. Wilting in the sun, if controlled carefully, can be a valuable adjunct to artificial drying of buckwheat for production of dried meals.

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Notes

A Note on a New Concentration of Antimony Trichloride Reagent in the U. S. P. XIV Identification Test for Vitamin A*

By JAMES A. BROWN

THE vitamin A identification test (Carr-Price test) which forms a part of the U. S. P. XIV assay for vitamin A (1) has been the subject of much study by many workers. The fleeting nature of the blue color formed is well known and constitutes a major obstacle to the utilization of this identification procedure.

To our knowledge, no work has been reported on studies of the reaction of vitamin A with concentrations of antimony trichloride reagent lower than specified.

Work in this laboratory has shown that the speed of the development to peak intensity of the vitamin A-$SbCl_3$ blue color can be decreased to a point where there is ample time to determine a readable maximum by use of a 6 Gm./100 cc. reagent instead of the 20 Gm./100 cc. Repeated determinations using both reagents have shown that the calibration curves obtained with the U. S. P. reference standard are similar in shape and degree of linearity, the difference being that as the reagent concentration decreases, the slope of the useful range decreases. Further, as long as the same reagent concentration is used for both standard and unknown, the results obtained for unknowns with either strength are identical except for variations in the results due to the inability to reproduce the readings as well with the stronger reagent as with the weaker.

Figure 1 shows the family of calibration curves resulting when various strengths of reagent are used, and Table I indicates the length of time required for the blue color to reach a maximum intensity when different strengths of reagent are used. Consideration of both the slope of the curve and the time data leads to the conclusion that the optimum reagent strength is about 6 Gm./100 cc. Strengths much below this give absorbancies below the 0.2 to 0.5 useful range specified by the U. S. P. XIV.

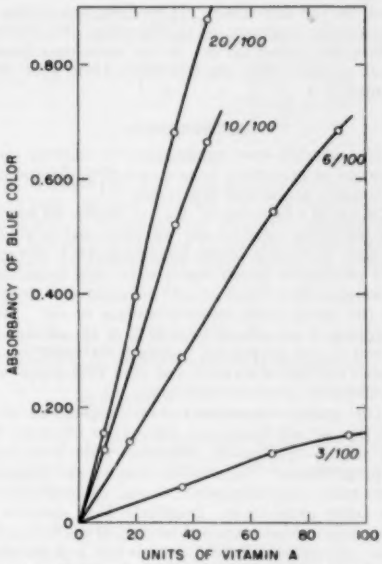

Fig. 1.—Calibration curves with various reagent strengths.

TABLE I

Strength of Reagent, Gm./100 Cc.	Time to Reach Peak Intensity, Sec.
20	1
10	3
6	7
3	13

When the weaker reagents are used, the characteristic red color which follows the blue color appears earlier. However, even with the weakest reagent, the red appears after the blue color maximum, and, so far as we have been able to determine, it has no absorption effect at 620 mμ. Both a modified Coleman Spectrophotometer and a Model B Beckman Spectrophotometer have been used in this laboratory for these measurements.

Other advantages of the weaker reagent are that it is less corrosive, making it easier to handle and dispense, and that it is less expensive to use.

It is hoped that other laboratories will gather data of their own using the weaker reagent, and that its general usefulness will become established to the end that it may become part of the official method.

* Received September 25, 1950, from the Analytical Laboratories, Eli Lilly and Co., Indianapolis, Ind.

REFERENCE

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A Note on the New U. S. P. XIV Method for Assay of Progesterone Injection*

By ERNEST J. UMBERGER

In the method described in U. S. P. XIV for the assay of progesterone injection, a sample equivalent to about 20 mg. of progesterone in a vegetable oil solution is added to a separator containing 40 cc. of petroleum benzin. The progesterone is extracted from this mixture with five successive 20-cc. portions of 90% alcohol. Because of the mutual solubility of petroleum benzin and 90% alcohol, only about 5 cc. of the petroleum benzin layer remains after the last extraction. It has been found that by the simple expedient of equilibrating the solvents before use, about 35 cc. of the petroleum benzin layer remains after the five extractions with 90% alcohol.

EXPERIMENTAL

The solvents were equilibrated by shaking equal volumes of petroleum benzin and 90% alcohol in a separatory funnel and separating.

In the first experiment, 1 cc. of sesame oil was extracted using equilibrated solvents, and a similar amount extracted using nonequilibrated solvents. The petroleum benzin layers were then transferred to weighed 50-cc. beakers and evaporated to dryness on the steam bath under a stream of air. After weighing, it was found that 89% of the oil was retained in the petroleum benzin layer when equilibrated solvents were used, but only 73% when nonequilibrated solvents were used.

The second experiment was designed to show whether or not there was any loss in efficiency by the use of equilibrated solvents in the extraction of progesterone. An alcohol solution was prepared containing approximately 20 mg. of progesterone per cubic centimeter. Duplicate 1-cc. samples of this solution were extracted by each of the two methods. As shown in Table I, sample nos. 1–4, identical results were obtained.

Finally, both methods were used for the assay of 0.8 cc. of a sesame oil solution containing 25 mg. of progesterone per cubic centimeter. These results are shown as sample nos. 5–8 in Table I. There appears to be a tendency toward high values when nonequilibrated solvents are used. This may be due to the incomplete removal of the oil residue from the 2,4-dinitrophenyl hydrazine derivatives, which is reflected in the melting points, also shown in Table I.

TABLE I.—RESULTS OF ANALYSES OF ALCOHOL AND SESAME OIL SOLUTIONS OF PROGESTERONE USING EQUILIBRATED AND NONEQUILIBRATED SOLVENTS IN THE U. S. P. XIV METHOD FOR PROGESTERONE INJECTION

Sample No.	Method	Mg. Found	M.p. of 2,4-Dinitrophenyl Hydrazine
	Alcohol Solution, 20 Mg./Cc. Used 1.0 Cc.		
1	Nonequilibrated	19.8	277–279°
2	Nonequilibrated	19.8	276–279°
3	Equilibrated	19.8	276–278°
4	Equilibrated	19.7	277–279°
	Oil Solution, 25 Mg./Cc. Used 0.8 Cc.		
5	Nonequilibrated	20.2	275–278°
6	Nonequilibrated	21.3	273–277°
7	Equilibrated	19.8	276–278°
8	Equilibrated	20.0	276–278°

DISCUSSION

It is apparent that the use of equilibrated solvents instead of nonequilibrated solvents in the U. S. P. XIV assay method for progesterone injection, results in a better separation of progesterone from its oily vehicle, and consequently in a purer 2,4-dinitrophenyl hydrazine derivative. Danger of mechanical loss is minimized since about 35 cc. of the petroleum benzin layer remains after the alcohol extractions using equilibrated solvents as compared to about 5 cc. using nonequilibrated solvents. This may be particularly important on a warm day when there would be greater loss of solvent due to evaporation. There is no decrease in the efficiency of the extraction of progesterone by the use of equilibrated solvents.

* Received November 4, 1950, from the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

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GERMAN JOURNALS MERGED

It has been announced that the *Sueddeutsche Apotheker-Zeitung* has merged with the *Deutsche Apotheker Zeitung*, and will be published under the name *Deutscher Apotheker Verlag*. The editor of the new publication will be Dr. Roland Schmiedel, former editor of the *Sueddeutsche Apotheker-Zeitung*. The address of the editorial office is Tuebinger Str. 53, Stuttgart S, Germany.

A Note on the Acute Toxicity of Neostigmine Methyl Bromide in the Rat*,†

By THOMAS J. HALEY and BONNIE M. RHODES‡, §

In the course of an investigation on inhibition and potentiation of autonomic nerve impulses to the gastrointestinal tract, it became necessary to determine the intravenous and subcutaneous acute toxicity of Neostigmine Methyl Bromide in the rat. Previous investigations (1–3) have been concerned almost entirely with such toxicity in mice, using the drug's methyl sulfate derivative.

The symptoms of acute toxicity observed were: fascicular twitching of the muscles, pupillary constriction, bloody lacrimation, diarrhea, and convulsions. Diarrhea occurred in all the animals within fifteen minutes. Chromodacryorrhea (bloody lacrimation) characteristic of cholinergic nerve stimulation or choline esterase inhibition (5, 6) was observed in all the animals.

Table I.—LD_{50}'s of Neostigmine Methyl Bromide in Rats

Mode of Administration	Total No. Animals	LD_{50}, Mg./Kg.	Range of LD_{50}, Mg./Kg., Odds 19/20	Slope	Range of Slope, Odds 19/20
Intravenous	25	0.165	0.152–0.179	1.12	0.91 –1.38
Subcutaneous	25	0.370	0.346–0.396	1.13	0.999–1.278

EXPERIMENTAL

Male CF1 rats weighing 80 to 175 Gm. (average 125 Gm.), in groups of five animals for each dosage level, were injected intravenously or subcutaneously with varying doses of Neostigmine Methyl Bromide. Table I gives the results obtained as calculated by the method of Litchfield and Wilcoxon (4).

* Received September 1, 1950, from the University of California, Atomic Energy Project, West Los Angeles, Calif.

† This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

‡ Division of Pharmacology and Toxicology, Atomic Energy Project, School of Medicine, University of California at Los Angeles.

§ The authors wish to thank Hoffmann-La Roche, Inc., for the Neostigmine Methyl Bromide used in this study.

SUMMARY

The intravenous and subcutaneous LD_{50}'s of Neostigmine Methyl Bromide for rats have been determined. The symptoms of acute toxicity in the rat are similar to those previously reported for the mouse with the methyl sulfate derivative of the drug.

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A Note on a Simple Respirator for Small Animals*

By L. D. EDWARDS and T. S. MIYA†

In the course of evaluating some new synthetic vasopressors in this laboratory, the Shipley and Tilden (1) pithed-rat preparation was used. The original technique employed as a respirator a modified, air-driven windshield wiper which could be adjusted to give a desired rate and volume output. These adjustments, of course, are necessary to obtain survival on one hand, and to avoid pulmonary edema on the other. It was suggested, in our work, that these respirator requirements could be met with another very simple device constructed of readily available laboratory apparatus. This device consists of a Metallic De Vilbiss Nozzle (No. 15) fixed to a board. A compressed air source is attached to the usual air inlet. The tubing which fits into the container when the atomizer is in normal use is connected to a T-tube which, in turn, is connected to the tracheal cannula. The T-tube should be as near as possible to the cannula, and the free arm of the tube equipped with a short piece of rubber tubing and a screw clamp. This clamp when regulated permits an adequate CO_2-O_2 exchange, and may be also used for volume control. The tracheal cannula should be short, and for rats in the 150–300-Gm. range, the size usually used, its tip diameter should be approximately 2 mm.

The nozzle-tip of the atomizer is fitted with a piece of rubber tubing which is placed between the arms of an ordinary 8-in. laboratory forcep held firmly to the board of the device. Interruption of air flow is executed by the action of a cork (No. 28) eccentrically mounted on an electric stirrer (Eber-

* Received May 5, 1950, from the School of Pharmacy, Purdue University, Lafayette, Ind.

Presented to the Scientific Section, A. Ph. A., Atlantic City meeting, May, 1950.

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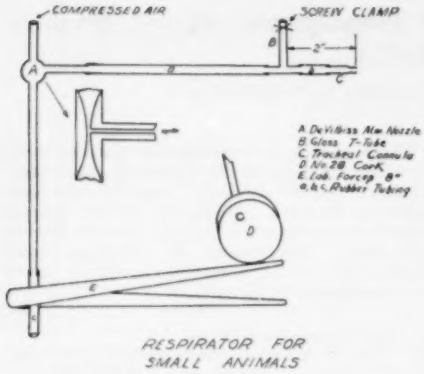

Figure 1.

bach's "Power-Stir" No. A77-836), the speed of which is regulated by a variac. When the nozzle opening is closed, the compressed air is shunted toward the animal; when it is open, the air passes over the opening at *A* (see Fig. 1) and provides adequate suction to empty the lungs (Venturi principle).

The volume output of the respirator may be regulated by lowering or raising the cork on the forcep or by regulating the screw clamp of the T-tube. Good thoracic expansion should simulate normal respiration. The rate is controlled by means of the variac. A rate of 65 to 70 per minute has been found to be optimum for rats of the 150–300-Gm. range. The color of the blood in the carotid artery is a suitable criterion for adequate oxygenation.

Pithed rats have been maintained with the respirator as described by several hours. As a rule, the respirator is connected and started just prior to pithing.

SUMMARY

A simple respirator for use in pithed rat preparations has been described.

REFERENCE

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1951 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1951 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1951 Award, nominations must be received on or before January 1, 1951.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, John C. Krantz, and Frank O. Taylor.

The award is now in its fourth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist, and the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University.

Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada, will receive the third award and will give the 1951 Iodine Award lecture.

Book Reviews

Biological Standardization. 2nd ed. By J. H. BURN, with chapters by D. J. FINNEY and L. G. GOODWIN. Oxford University Press, New York, 1950. x + 440 pp. Illustrated. 14 x 23.5 cm. Price $6.75.

Pharmacologists will be glad to see a new edition of this book, the methods of the first edition having been largely replaced or improved upon to such an extent that it had almost lost its usefulness. This new edition lacks the completeness of coverage of the old edition, though this is understandable when one compares the total volume of pharmacological literature of the two periods. The book retains, however, its full value as an introductory text for biological assay, and the methods that are included are, for the most part, modern. Readers will be pleased to see the chapter, "Mathematical Treatment of Results," replaced by a chapter of one-hundred and fifty pages entitled, "Statistical Analysis." Pharmacologists will be particularly pleased to see that examples used in this chapter, and they are numerous, are practical bioassay problems.

Also of special interest are well-described methods for biological assay of local anesthetics, drugs affecting gastric secretion, quinidine-like compounds, curariforms, anthelmintics, and other newer types of drugs which are still in developmental stages.

Many workers in this country will be disappointed that the second edition does not cover adequately the developments in bioassay of digitalis and similar drugs.

Though this book does not have the completeness of the first edition at its time of publication, the second edition remains a valuable tool to the pharmacologist and bioassayist because of the thorough treatment given those subjects that it does include.

The Essential Oils. Vol. IV. By ERNEST GUENTHER. D. Van Nostrand Company, Inc., New York, 1950. xiv + 752 pp. Illustrated. 15 x 23.5 cm. Price $12.

The fourth volume of this monumental series of books on the essential oils was published in October. Volume I, published in 1948, covered the history, chemistry, biology, origin, functions, methods of production, and analyses of essential oils from a general point of view [THIS JOURNAL, **37**, 214(1948)]. In the second volume, published in 1949, the constituents of essential oils including the hydrocarbons, the terpenes, the sesquiterpenes and their oxygenated derivatives, were discussed quite in detail from the standpoint of occurrence, isolation, identification, properties, and use [THIS JOURNAL, **38**, 665(1949)]. Volume III, also published in 1949, was devoted to monographs on essential oils from members of the *Rutaceae* and *Labiatae* plant families [THIS JOURNAL, **39**, 243(1950)]. Volume IV continues according to the design established for the third volume and covers the essential oils obtained from the members of the *Gramineae*, *Lauraceae*, *Burseraceae*, *Myrtaceae*, *Umbelliferae*, and *Geraniaceae* families. As in the previous volume, each monograph covers the history, geographic origin, botanical description, methods of cultivation of the plant from which the oil is derived, techniques of production and yield of oil, physical and chemical properties, chemical compositions, and use in the industry.

With the appearance of each succeeding volume of *The Essential Oils* comes a greater appreciation of the magnitude of the task undertaken by Ernest Guenther when he started to write a comprehensive series of books covering the essential oils. He and his associates are to be congratulated upon the success of the undertaking thus far in its progress toward completion. All who are interested in essential oils from any standpoint will look forward with keen anticipation to the appearance of the succeeding volume which is promised for future publication.

Cell Physiology and Pharmacology. By J. F. DANIELLI. Elsevier Publishing Company, Inc., New York, 1950. viii + 156 pp. Illustrated. 15.5 x 23.5 cm. Price $3.

This book, based upon a series of lectures given at University College, London, is intended to stimulate more effective thought in the planning of research on new drugs. The author recognizes the great need that exists today for some logical approach to the development of new drugs which will make it possible in the future to avoid the synthesis of such large numbers of compounds in order to find one that is useful. He recognizes as well the tremendous loss of effort on the part of the pharmacologists who must make series of tests on thousands of chemicals that are without value to medicine. In attempting to achieve his task, the author presents in a well-integrated fashion the various theories of cell structure and physiology which have to do with drug action, instructing that these theories and mechanisms serve as a basis of thought for planning new drugs.

Although a number of books on the mechanism of drug action have appeared recently, this one has a definite place of its own. It presents a broader view of the subject than do most books, with less detail in most subjects, it is true, but in a more thought-provoking manner. At the same time, it is remarkably easy to follow—the type of concise writing which adds one simple point to another, piling them up until some general conclusions can be drawn. At the same time oversimplifications are few in number.

The subject is begun with a consideration of the structure of the cell, then the biological changes of the cell during growth, the structure of chromosomes and the relative sizes and quantities of cell constit-

uents. The physicochemical properties of cells and cell contents are described. The reviewer does find it disconcerting, however, to see biochemical reactions involving foreign substances, so-called detoxication reactions, classified as defense mechanisms of cells.

The chapter called "Possible Actions of Drugs on Surfaces" takes up the theories of drug action based on ion-dipole and dipole-dipole reactions. Included are some possible explanations of lytic action, of the activity of stilbene derivatives, and the action of quaternary salts. Membrane permeability effects are also presented. Action of drugs on enzymes is presented but with much less emphasis than will satisfy the modern researcher. A very commendable section is then devoted to mitotic poisons, nuclear and cytoplasmic drug action, and drug action upon genes. This section furnishes an excellent introductory background for the more recent approaches in cancer research.

This book will be pleasurable and easy reading for workers already engaged in pharmaceutical and medical research, and it will be of much more value to newcomers to the field. Its greatest usefulness will be to the student and the teacher. It is especially worthy of the attention of anyone planning graduate courses for pharmaceutical students.

Penicillin—Its Practical Application. 2nd ed. By SIR ALEXANDER FLEMING. C. V. Mosby Company, St. Louis, 1950. xiii + 491 pp. Illustrated. 13.5 x 22 cm. Price $7.

Since the time of appearance of the first edition of this book, about four years ago, much more of the practical use and value of penicillin has become known. Moreover, new forms and purer material have come into use, so that this second edition is a timely publication.

This book contains a very well constructed and not too long review of the history of the development of penicillin, its chemisty and its manufacture. Simplified flow sheets are included so that the reader can see the manufacturing process at a glance.

The pharmacist will be interested to find a summary of incompatibilities of penicillin, and precautions for mixing and dispensing. Formulas for penicillin creams, oil injection and aqueous injection of the British Pharmacopœia and other dosage forms not official, such as eye drops, tablets, insufflation powders, and inhalations are included.

The pharmacology of penicillin is brought up-to-date in this edition, and this includes a discussion of absorption through various organs. Methods of administration are discussed in some detail, and special apparatus is illustrated.

The largest portion of the book describes the therapeutic use of penicillin in various diseased conditions and for prophylaxis. Case reports are included throughout this section to illustrate the type of results that have been obtained, as well as to warn against incorrect usage. Veterinary use of penicillin is also described.

There is a chapter included on penicillin sensitivity, a subject which is increasingly important as the use of penicillin becomes more widespread. Tests for sensitivity are described and their significance noted, while methods for treating or overcoming sensitivity are presented.

This review of the practical application of penicillin should prove to be a valuable reference for the practicing pharmacist and offers an opportunity to bring one's knowledge of penicillin therapy and dispensing up-to-date.

Organophosphorus Compounds. By GENNADY M. KOSOLAPOFF. John Wiley and Sons, Inc., New York, 1950. vii + 376 pp. 15 x 23.5 cm. Price $7.50.

In the preface to this book the author states that it was written in the hope of filling a significant gap in the chemical literature, and to present the general aspects of the organic chemistry of phosphorus. An examination of the text of the book is convincing proof that the author has been quite successful. The physical and chemical properties of all known organophosphorus compounds are covered. Methods of synthesis for each of the various classes of these compounds are included.

Each chapter of *Organophosphorus Compounds* covers a separate class, beginning with phosphines and related compounds and including halophosphines; halophosphine halides and phosphonyl halides; quaternary phosphonium compounds; tertiary phosphine oxides, sulfides, and selenides; phosphinous, phosphonous, and phosphonic acids, their sulfur analogs and esters; phosphites and thiophosphites; phosphates, halophosphates, and thio analogs; compounds with phosphorus to nitrogen bonds; quasi-phosphonium compounds; and derivatives of anhydro acids.

Each chapter follows the same general pattern. A brief introductory statement is followed by a compilation of the methods of synthesis applicable to the class of compounds being discussed. The synthetic section of each chapter is followed by a summary of the general physical and chemical features of the particular class. A list of all known compounds in a given class is included in each chapter, and a reference to a general method of synthesis in another part of the chapter is given for each compound listed. The book is well documented throughout and includes all references to the organic chemistry of phosphorus compounds that have appeared in the chemical literature up to January, 1950.

Antipyrine: A Critical Bibliographic Review. By LEON A. GREENBERG. Hillhouse Press, New Haven, Conn., 1950. xvi + 135 pp. 16 x 24.3 cm. Price $4.

This monograph is the third in a series sponsored by the Institute for the Study of Analgesic and Sedative Drugs. The first of the series, a monograph on *Acetanilid*, was published in 1946 [THIS JOURNAL, **35**, 351(1946)]. The second of the series covering *The Salicylates* was published in 1948 [THIS JOURNAL, **38**, 57(1949)]. Both of the first two monographs of the series were favorably reviewed in THIS JOURNAL.

In the recently published monograph on *Antipyrine*, the same high standards established in the two preceding reports of the series have been maintained. The topics discussed in the monograph are the history, physical and chemical properties; the fate of antipyrine in the body; pharmacology

and toxicology of antipyrine; therapeutic uses; antipyrine poisoning; and tolerances, addiction and habituation. An idea of the thoroughness with which the literature has been searched for information relating to antipyrine is obtained from the 1,735 item bibliography and author index, which constitute more than half the book.

Antipyrine, originally used exclusively as a fever-reducing drug, is one of the first important synthetic drugs introduced into medicine. Following the general decline in the use of antipyretic agents, antipyrine continued to be used for its analgesic action. Of the many pyrazolon compounds that have been used in medicine in the past, antipyrine and aminopyrine are the main survivors. During recent years neither antipyrine nor aminopyrine has been extensively prescribed. The Prescription Study of the Pharmaceutical Survey reports a frequency range of 120 for the former, and 149 for the latter. The author expresses the opinion that "the present neglect of antipyrine as an effective analgesic appears to represent merely a vagary of fashion—perhaps occasioned by the popularity of salicylate." The extensive data presented from the comprehensive literature survey reported, and the analysis of the data by the author tend to indicate that antipyrine is an effective analgesic and that there is no justification in the experimental and clinical literature for its declining use in medicine. The Institute for the Study of Analgesic and Sedative Drugs is performing a useful service to research workers in biochemistry, pharmacology, and also to clinicians, in compiling extensive bibliographies on drugs such as acetanilid, antipyrine, and the salicylates. Other volumes now in preparation will cover bromides and phenacetin.

A German-English Dictionary for Chemists. 3rd ed. By AUSTIN M. PATTERSON. John Wiley and Sons, Inc., New York, 1950. xviii + 541 pp. 13 x 17.5 cm. Price $5.

Patterson's *German-English Dictionary for Chemists* has no counterpart. Since the appearance of the first edition in 1917 it has served as a useful aid to pharmaceutical chemists in this country in reading technical reports written in the German language. Because this dictionary is so well known, there is little to be said about the third edition with which all who have had occasion to use one of the previous editions are not already familiar.

The third edition continues to include the same useful features of previous editions, such as the introduction giving hints for using the dictionary and explaining peculiarities of German chemical nomenclature, a general vocabulary making a second dictionary unnecessary, and many other features which have made the book so successful and so valuable through the years since the appearance of the first edition.

The number of entries has been increased from 42,000 in the second edition to 59,000 in the third edition. Professor Patterson's *German-English Dictionary for Chemists* cannot be too highly recommended for use by anyone requiring the use of a dictionary to read scientific articles written in the German language.

Colloid Chemistry, Theoretical and Applied. Vol. VII. Edited by JEROME ALEXANDER. Reinhold Publishing Corporation, New York, 1950. xi + 736 pp. Illustrated. 15 x 23.5 cm. Price $15.

This volume includes 43 papers on theory and methods, biology and medicine, and technology. The book is divided into three parts. Part I, entitled Theory and Methods, covers the basic structure and behavior of matter at various structural levels. Part II covers biology and medicine, and includes papers relating to proteins, enzymes, drugs, genetics, tuberculosis, blood types, and dental caries. This section undoubtedly is of greater pharmaceutical interest than either of the other two parts. Of especial pharmaceutical interest is the chapter by Frederick Bernheim on the interaction of drugs and enzymes. Part III is devoted to technological applications and includes papers on such widely varied subjects of pharmaceutical interest as detergents, cosmetics, protective wrappings, packaging, and freeze-drying.

The Petroleum Chemicals Industry. By RICHARD FRANK GOLDSTEIN. John Wiley and Sons, Inc., New York, 1950. xiii + 449 pp. Illustrated. 15.5 x 25 cm. Price $8.50.

In this book the fields of industrial chemistry in which petroleum is the starting material are surveyed and summarized. The author has also, in some instances, included information on competitive processes and information relating to methods of production from petroleum which are not the most economical ones but which may, with certain improvements, become of economic importance.

Chapter 1 covers the sources of petroleum hydrocarbons present in crude petroleum or produced as by-products in the refining operations of the petroleum industry. Chapters 2 to 5 are devoted to the chemistry of paraffins, chapters 6 to 10 cover the manufacture of olefins and the chemical developments dependent on them. The manufacture of other classes of hydrocarbons such as the diolefins, naphthenes, aromatics, acetylene, aldehydes, and ketones are covered in chapters 11 to 19. In chapter 20 a summary of the chemical by-products is presented. The by-products covered in this chapter are usually nonhydrocarbons in nature, which are obtained from refinery operations. The book is well documented throughout and the typography and binding help to make a very readable book.

BOOKS RECEIVED

Chymia: Annual Studies in the History of Chemistry. Vol. 3. HENRY M. LEICESTER, Editor-in-Chief. University of Pennsylvania Press, Philadelphia, 1950. ix + 251 pp. Illustrated. 15.5 x 24 cm. Price $4.50.

Die Tablettenfabrikation und ihre maschinellen Hilfsmittel. 5th ed. By JOHANNES ARENDS. Springer-Verlag, Berlin, 1950. 262 pp. Illustrated. 15 x 21 cm. Price DM 25.50.

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Antihistamines: Industry and Product Survey. By NATHAN WISHNEPSKY. Chemonomics, Inc., New York, 1950. 157 pp. 15.5 x 23 cm. Price $5.

Organophosphorus Compounds. By GENNADY M. KOSOLAPOFF. John Wiley and Sons, Inc., New York, 1950. vii + 376 pp. 15 x 23.5 cm. Price $7.50.

Colorimetric Determination of Traces of Metals. 2nd ed. By E. B. SANDELL. Interscience Publishers, Inc., New York, 1950. xix + 673 pp. 15 x 23.5 cm. Price $9.

Antipyrine: A Critical Bibliographic Review. By LEON A. GREENBERG. Hillhouse Press, New Haven, Conn., 1950. xvi + 135 pp. 16 x 24.3 cm. Price $4.

Analytical Absorption Spectroscopy. Edited by M. G. MELLON. John Wiley and Sons, New York, 1950. vii + 618 pp. Illustrated. 15 x 23.5 cm. Price $9.

Plant Viruses and Virus Diseases. 3rd ed. By F. C. BAWDEN. The Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York City, 1950. xiv + 335 pp. Illustrated. 17 x 27 cm. Price $9.

Physiology and Anatomy. 6th ed. By ESTHER M. GREISHEIMER. J. B. Lippincott Company, Philadelphia, 1950. xvi + 841 pp. Illustrated. 14 x 21 cm. Price $4.

Organic Syntheses. Vol. 30. ARTHUR C. COPE, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1950. vi + 115 pp. 15 x 23.5 cm. Price $2.50.

Jan Ingenhousz, Plant Physiologist (With a History of the Discovery of Photosynthesis). By HOWARD S. REED. Reprinted from the Chronica Botanica, Vol. 11, No. 5/6, pp. 285–396. The Chronica Botanica Co., Waltham, Mass.; Stechert-Hafner, Inc., New York City. Illustrated. 17.5 x 26 cm. Price $3.

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Prologue to Tomorrow. By ROBERT KEITH LEAVITT. The Pennsylvania Salt Manufacturing Company, Philadelphia, 1950. 100 pp. Illustrated. 15.5 x 23.5 cm.

Colloid Chemistry, Theoretical and Applied. Vol. VII. Edited by JEROME ALEXANDER. Reinhold Publishing Corporation, New York, 1950. xi + 736 pp. Illustrated. 15 x 23.5 cm. Price $15.

The Petroleum Chemicals Industry. By RICHARD FRANK GOLDSTEIN. John Wiley and Sons, Inc., New York, 1950. xiii + 449 pp. Illustrated. 15.5 x 25 cm. Price $8.50.

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The Pharmaceutical Industry in Germany During the Period 1939–1945. By J. M. COPPOCK. Published for the British Intelligence Objectives Sub-Committee by His Majesty's Stationery Office, London, 1950. 120 pp. Illustrated. 15 x 24.5 cm. Price 90¢. (Available from British Information Services; 30 Rockefeller Plaza; New York 20, N. Y.)

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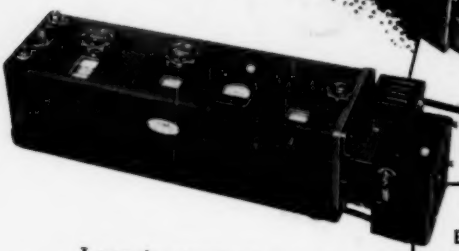

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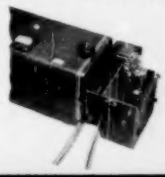

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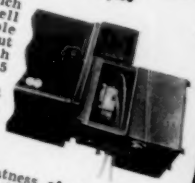

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